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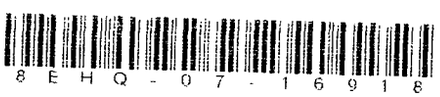
August 09, 2007



TSCA Confidential Business Information Center (7407M)
EPA East - Room 6428 Attn: Section 8(e)
United States Environmental Protection Agency
1200 Pennsylvania Avenue, NW
Washington DC 20460-0001

CONTAIN NO CBI

Attention: TSCA 8(e) Coordinator

RE: Tertiary-Butyl Acetate – Alga Inhibition Test

Dear Sir or Madam:

Lyondell Chemical Company (Lyondell) hereby submits this letter pursuant to Section 8(e) of the Toxic Substances Control Act (TSCA) and EPA's 1991 Section 8(e) Reporting Guide because it includes findings that EPA may consider reportable. Lyondell has not made a determination as to whether a significant risk of injury to health or the environment is actually presented by these findings.

Tertiary-Butyl Acetate was tested to assess its effect on the growth of the green algae *Pseudokirchneriella subcapitata*. The test followed the method described in the OECD Guidelines for Testing of Chemicals (1984) No 201, "Alga, Growth Inhibition Test" plus the relevant EPA guidelines (including CFR 40 Part 797 Section 1050 and Draft OPPTS 850.5400), and exposure to tertiary-butyl acetate gave the following results:

- 72-hour E_rC_{50} (growth rate) value of 530 mg/L based on added test material (96-hour value 670 mg/l)
- 72-hour E_rC_{50} (growth rate) value of 16 mg/L based on mean, measured test concentrations (96-hour value 64 mg/l)
- 72-hour E_bC_{50} (biomass) value of 220 mg/L based on added test material (96-hour value 210 mg/l)
- 72-hour E_bC_{50} (growth rate) value of 6.1 mg/L based on mean, measured test concentrations (96-hour value 32 mg/l)
- NOEC value of 32 mg/L based on added test material (after 72 and 96 hours)

- NOEC value of 2.3 mg/L based on mean, measured test concentrations (after 72 and 96 hours).

The test was performed in closed vessels to minimize test material loss by volatilization. Exposure levels were confirmed by GC analysis at the start of the test and after 96 hours. At all but the highest tested concentration (1000 mg/l), no tertiary-butyl acetate was detected at the end of the test period. This was shown to be related to hydrolysis, forming tertiary-butyl alcohol. The measured concentration EC_{50} and NOEC values shown above were therefore calculated using a worst-case model based on geometric means of initial and final values (96-hour concentrations taken as one half of the limit of analytical quantification, except at nominal 1000 mg/l). For this reason, results based on measured concentrations appear low relative to those based on nominal concentrations.

A copy of the study final report is enclosed.

Should you have any questions or require additional details, please do not hesitate to call me at 713-309-7145. I may also be reached by e-mail at lisa.wells@lyondell.com.

Sincerely,

Lisa S. Wells

Lisa S. Wells
Corporate TSCA Coordinator
Lyondell Chemical Company

Enclosure

Cc: TSCA 8(e) Files

SafePharm Laboratories

tert-Butyl acetate:

ALGAL INHIBITION TEST

SPL PROJECT NUMBER: 2197/0026

OECD GUIDELINE NO. 201; US CODE OF FEDERAL REGULATIONS TITLE 40, PART 797, SECTION 1050; US EPA PESTICIDE ASSESSMENT GUIDELINES, SUB-DIVISION J, SECTION 122-2 AND US EPA DRAFT ECOLOGICAL EFFECTS TEST GUIDELINE OPPTS 850.5400

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QUALITY ASSURANCE REPORT

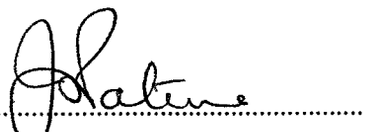
This study type is classed as short-term. The standard test method for this study type ("General Study Plan" in OECD terminology) was reviewed for compliance once only on initial production. Inspection of the routine and repetitive procedures that constitute the study is carried out as a continuous process designed to encompass the major phases at or about the time this study was in progress.

This report has been audited by Safepharm Quality Assurance Unit, and is considered to be an accurate account of the data generated and of the procedures followed.

In each case, the outcome of QA evaluation is reported to the Study Director and Management on the day of evaluation. Audits of study documentation, and process inspections appropriate to the type and schedule of this study were as follows:

	17 May 2006	Standard Test Method Compliance Audit
	05 June 2007	Test Material Preparation
	05, 19 June 2007	Test System Preparation
	05, 19 June 2007	Exposure
	27 June 2007	Assessment of Response
	15, 25 June 2007	Chemical Analysis
§	16 July 2007	Draft Report Audit
§	Date of QA Signature	Final Report Audit

§ Evaluation specific to this study



..... DATE: 18 JUL 2007

For Safepharm Quality Assurance Unit*

***Authorised QA Signatures:**

Head of Department:	JR Pateman CBiol MIBiol DipRQA ACQI FRQA
Deputy Head of Department:	JM Crowther MIScT MRQA
Senior Audit Staff:	JV Johnson BSc MRQA; G Wren ONC MRQA

GLP COMPLIANCE STATEMENT

The work described was performed in compliance with UK GLP standards (Schedule 1, Good Laboratory Practice Regulations 1999 (SI 1999/3106 as amended by SI 2004/0994)). These Regulations are in accordance with GLP standards published as OECD Principles on Good Laboratory Practice (revised 1997, ENV/MC/CHEM(98)17); and are in accordance with, and implement, the requirements of Directives 2004/9/EC and 2004/10/EC.

These international standards are acceptable to the Regulatory agencies of the following countries: Australia, Austria, Belgium, Canada, the Czech Republic, Denmark, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Israel, Italy, Japan, Republic of Korea, Luxembourg, Mexico, The Netherlands, New Zealand, Norway, Poland, Portugal, Slovenia, South Africa, Spain, Sweden, Switzerland, Turkey, the United Kingdom, and the United States of America.

This report fully and accurately reflects the procedures used and data generated.

H. Vryenhoef ..... Date: 18 JUL 2007

H Vryenhoef BSc
Study Director

The analytical data presented in this report were compiled by me or under my supervision and accurately reflect the data obtained.

..... Date: 18 JUL 2007

D M Mullee CChem MRSC
Director of Analytical Services

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SUMMARY

Introduction. A study was performed to assess the effect of the test material on the growth of the green alga *Pseudokirchneriella subcapitata.* The method followed that described in the OECD Guidelines for Testing of Chemicals (1984) No 201, "Alga, Growth Inhibition Test" referenced as Method C.3 of Commission Directive 92/69/EEC (which constitutes Annex V of Council Directive 67/548/EEC), US CFR Title 40 Part 797 Section 1050, US EPA Pesticide Assessment Guidelines, Sub-Division J, Section 122-2 and the US EPA Draft Ecological Effects Test Guideline OPPTS 850.5400.

Methods. Preliminary trials with chemical analysis (establishing analytical method, assessing stability and test mixture formulation) found that levels of tert-butyl acetate in aqueous medium fell markedly over 96 hours due to volatilisation from open vessels and in closed vessels levels also fell, due to abiotic degradation (hydrolysis). Initial concentrations were also reduced in the presence of algal cells, suggesting adsorption onto cell surfaces.

Following a range-finding test, a definitive test using *Pseudokirchneriella subcapitata* was performed using aqueous solutions of the test material at nominal concentrations of 10, 32, 100, 320 and 1000 mg/l (three replicate flasks per concentration) for 96 hours, under constant illumination and shaking at a temperature of 24 ± 1°C.

To control losses by volatilization, testing was conducted in completely filled, stoppered test vessels. Following the recommendations of published data (Herman *et al* 1990) in order to prevent inhibition of growth due to the restriction of gaseous exchange, additional sodium bicarbonate was added to the culture medium to provide a source of carbon dioxide for algal growth. Samples of the algal populations were removed daily and cell concentrations determined for each control and treatment group, using a Coulter® Multisizer Particle Counter.

Results. Exposure of Pseudokirchneriella subcapitata to the higher concentrations of test material employed produced evidence of algal growth inhibition: calculated EC_{50} and NOEC values are shown in the following table. Those based on nominal test concentrations reflect the effect of test material added into test vessels, while those based on mean, measured concentrations reflect a worst-case model of rapid test material removal from test mixtures.

Parameter (with 95% confidence limits)		72-Hour value (mg/l)	96-Hour value (mg/l)
E_rC_{50} (growth rate)	Based on added test material	530 (420-670)	670 (limits not calculable)
	Based on mean, measured test concentrations*	16 (11-23)	64 (limits not calculable)
E_bC_{50} (biomass)	Based on added test material	220 (180-270)	210 (160-280)
	Based on mean, measured test concentrations*	6.1 (5.4-6.9)	5.8 (4.9-6.9)
NOEC	Based on added test material	32	32
	Based on mean, measured test concentrations*	2.3	2.3

In the definitive test (using closed vessels), only vessels to which test material had been added at 320 or 1000 mg/l still contained measurable test material concentrations after 96 hours (14 or 76% of nominal values respectively): the worst-case model cited above was therefore applied to give results based on measured concentrations.

Conversion of tert-butyl acetate to its tert-butyl alcohol hydrolysis product was confirmed by chemical analysis: measured concentrations of this hydrolysis product increased from <3 mg/l (the limit of quantitation) at 0 hours to 64-78% of maximum theoretical content (for 100% test material hydrolysis) at 96 hours except at the highest test concentration, where the extent of hydrolysis appeared to be less. Thus algal cells were exposed to increasing levels of the alcohol as tert-butyl acetate concentrations fell.

Exposure of *Pseudokirchneriella subcapitata* to the reference material zinc chloride gave a 96-hour E_rC_{50} value of 0.39 mg/l, a 96-hour, E_bC_{50} value of 0.17 mg/l and a NOEC of 0.10 mg/l.

* Worst-case model: approach cited in the OECD Guidance Document on Aquatic Testing of Difficult Substances and Mixtures for unstable test materials. Geometric means of initial and final measured concentrations of tert-butyl acetate (0h: 68-83% of nominal. 96h: below the limit of analytical quantitation, so taken as 0.5x this, i.e. 0.22 mg/l, with the exception of the 1000 mg/l test concentration which gave a measured concentration of 49% of nominal).

tert-Butyl acetate:

ALGAL INHIBITION TEST

1. INTRODUCTION

This report contains a description of the methods used and results obtained during a study to investigate the effect of the test material on the growth of the green alga *Pseudokirchneriella subcapitata*. The method followed that described in the OECD Guidelines for Testing of Chemicals (1984) No 201, "Alga, Growth Inhibition Test" referenced as Method C.3 of Commission Directive 92/69/EEC (which constitutes Annex V of Council Directive 67/548/EEC), US CFR Title 40 Part 797 Section 1050, US EPA Pesticide Assessment Guidelines, Sub-Division J, Section 122-2 and the US EPA Draft Ecological Effects Test Guideline OPPTS 850.5400.

The test material was suspected to be volatile and hence testing was conducted in completely filled, stoppered test vessels in order to minimise possible losses due to volatilisation. Following the recommendations of published data (Herman *et al* 1990 and Mayer *et al* 2000) in order to prevent inhibition of growth due to the restriction of gaseous exchange, additional sodium bicarbonate was added to the culture medium to provide a source of carbon dioxide for algal growth.

Pseudokirchneriella subcapitata is a freshwater unicellular alga, representative of primary producers found in natural waters and can therefore be considered as an important non-target organism in freshwater ecosystems.

The study was conducted between 13 April 2007 and 4 July 2007

The positive control (Safepharm Laboratories Project Number: 0039/0883) was conducted between 30 October 2006 and 3 November 2006.

2. TEST MATERIAL

2.1 Description, Identification and Storage Conditions

Sponsor's identification	:	tert-Butyl acetate
Description	:	clear, colourless liquid
Batch number	:	OD61002001
Date received	:	22 March 2007
Storage conditions	:	room temperature in the dark

The integrity of supplied data relating to the identity, purity and stability of the test material is the responsibility of the Sponsor.

3. METHODS

3.1 Test Species

The test was carried out using *Pseudokirchneriella subcapitata* strain CCAP 278/4. Liquid cultures of *Pseudokirchneriella subcapitata* were obtained from the Culture Collection of Algae and Protozoa (CCAP), Dunstaffnage Marine Laboratory, Oban, Argyll, Scotland. Cultures were maintained in the laboratory by the periodic replenishment of culture medium (Section 3.2). The culture was maintained in the laboratory at a temperature of 21 ± 1°C under continuous illumination (intensity approximately 7000 lux) and constant aeration.

3.2 Culture Medium

The culture medium used for the definitive test was the same as that used to maintain the stock culture however additional sodium bicarbonate (500 mg/l) was added after preparation in order to provide a source of CO_2 required for algal growth in sealed test vessels.

The culture medium is defined in Appendix 1.

3.3 Procedure

3.3.1 Range-finding test

The test concentrations to be used in the definitive test were determined by a preliminary range-finding test. The range-finding test was conducted by exposing *Pseudokirchneriella subcapitata* cells to a series of nominal test concentrations of 1.0, 10, 100 and 1000 mg/l for a period of 96 hours.

The test was conducted in 250 ml glass conical flasks completely filled with test preparation and sealed with ground glass stoppers to reduce losses due to volatility. Two replicate flasks were prepared for each control and test concentration. The test material was dissolved directly in culture medium.

An amount of test material (1000 mg) was dissolved in culture medium and the volume adjusted to 1 litre to give a 1000 mg/l stock solution from which serial dilutions were made to give further stock solutions of 100, 10 and 1.0 mg/l. An aliquot (900 ml) of each stock solution was

inoculated with algal suspension (4.5 ml) to give the required test concentrations of 1.0, 10, 100 and 1000 mg/l.

The stock solutions and each of the prepared concentrations were inverted several times to ensure adequate mixing and homogeneity.

The control group was maintained under identical conditions but not exposed to the test material.

At the start of the range-finding test a sample of each test and control culture was removed and the cell density determined using a Coulter® Multisizer Particle Counter. The flasks were then sealed with ground glass stoppers and incubated (INFORS Multitron® Version 2 incubator) at 24 ± 1°C under continuous illumination (intensity approximately 7000 lux) and constantly shaken at approximately 150 rpm for 96 hours.

After 96 hours the cell density of each flask was determined using a Coulter® Multisizer Particle Counter.

3.3.2 Definitive test

Based on the results of the range-finding test the following test concentrations were assigned to the definitive test: 10, 32, 100, 320 and 1000 mg/l.

3.3.2.1 Experimental Preparation

For the purpose of the definitive test, the test material was dissolved directly in culture medium.

Amounts of test material (640 and 2000 mg) were each separately dissolved in culture medium with the aid of ultrasonication for approximately 5 minutes and the volume adjusted to 2 litres to give 320 and 1000 mg/l stock solutions. Serial dilutions were then made from these to give further stock solutions of 100, 32 and 10 mg/l. An aliquot (1500 ml) of each stock solution was inoculated with algal suspension (7.5 ml) to give the required test concentrations of 10, 32, 100, 320 and 1000 mg/l.

The stock solutions and each prepared concentration were inverted several times to ensure adequate mixing and homogeneity.

The concentration and stability of the test material in the test preparations were verified by chemical analysis at 0 and 96 hours (see Appendix 1). In addition, one extra replicate vessel for

the control and each test concentration was prepared at 0 hours without the addition of algal cells and incubated alongside the definitive test to provide samples for chemical analysis at 96 hours from vessels which had not been opened over the test period.

3.3.2.2 Exposure conditions

As in the range-finding test 250 ml glass conical flasks were used. Three flasks each completely filled with test preparation were used for the control and each treatment group.

The control group was maintained under identical conditions but not exposed to the test material.

Pre-culture conditions gave an algal suspension in log phase growth characterised by a cell density of 5.64×10^6 cells per ml. This suspension was diluted to a cell density of 1.99×10^6 cells per ml prior to use. Inoculation of 1500 ml of test medium with 7.5 ml of this algal suspension gave an initial cell density of 1×10^4 cells per ml and had no significant dilution effect on the final test concentration.

The flasks were sealed with ground glass stoppers and incubated (INFORS Multitron® Version 2 incubator) at 24 ± 1°C under continuous illumination (intensity approximately 7000 lux) and constantly shaken at approximately 150 rpm for 96 hours.

Samples were taken at 0, 24, 48, 72 and 96 hours and the cell densities determined using a Coulter® Multisizer Particle Counter.

3.3.2.3 Physico-chemical measurements

The pH of each control and test flask was determined at initiation of the test and after 96 hours exposure. The pH was measured using a WTW pH 320 pH meter. The temperature within the incubator was recorded hourly using the incubator's internal pt-100 temperature probe and Multicom Software package (see Figure 10).

3.3.2.4 Verification of test concentrations

Samples were taken from the control (replicates R_1 - R_3 pooled) and each test group (replicates R_1 - R_3 pooled) at 0 and 96 hours for quantitative analysis. Samples were also taken for quantitative analysis at 96 hours from the test vessels prepared without the addition of algal cells. Duplicate samples were taken at 0 and 96 hours and stored at approximately -20°C for further analysis if necessary.

The method of analysis, stability, recovery and test preparation analyses are described in Appendix 2.

3.3.3 Evaluation of data

3.3.3.1 Comparison of areas under the growth curves

The area under the curve is taken to be an index of growth and was calculated using the following equation:

$$A = \frac{N_1 - N_0}{2} \times t_1 + \frac{N_1 + N_2 - 2N_0}{2} \times (t_2 - t_1) + \frac{N_{n-1} + N_n - 2N_0}{2} \times (t_n - t_{n-1})$$

where

A	=	area
N_0	=	cell concentration at the start of the test
N_1	=	cell concentration at t_1
N_n	=	cell concentration at t_n
t_1	=	time of first measurement (hours from start)
t_n	=	time of nth measurement (hours from start)

Percentage inhibition of growth at each test concentration (I_A) was calculated by comparing the area under the test curve (A_t) with that under the control curve (A_c) using the following equation:

$$I_A = \frac{A_c - A_t}{A_c} \times 100$$

The percentage inhibition values (I_A) were plotted against test concentration, a line fitted using the Xlfit software package (IDBS) and the EC_{50} values with respect to the area under the growth curve (biomass) E_bC_{50} (72 and 96 h), determined from the equation for the fitted line.

3.3.3.2 Comparison of growth rates

The average maximum growth rate (μ) for each culture was also calculated, from the straight section of the growth curve (Figure 1), using the following equation:

$$\mu = \frac{\ln N_n - \ln N_1}{t_n - t_1}$$

Percentage inhibition of growth rate at each test concentration (I_μ) was calculated by comparing the growth rate (μ_t) with that of the control (μ_c) using the following equation:

$$I_\mu = \frac{\mu_c \quad \mu_t}{\mu_c} \times 100$$

The percentage inhibition values (I_μ) were plotted against test concentration, a line fitted using the Xlfit software package (IDBS) and the EC_{50} values with respect to the growth rate, E_rC_{50} (0 – 72 and 0 – 96 h), determined from the equation for the fitted line.

3.3.3.3 95% Confidence limits

The 95% confidence limits were calculated using the method of Litchfield and Wilcoxon (Litchfield and Wilcoxon 1949).

3.3.3.4 Statistical analysis

One way analysis of variance incorporating Bartlett's test for homogeneity of variance (Sokal and Rohlf 1981) and Dunnett's multiple comparison procedure for comparing several treatments with a control (Dunnett 1955) was carried out on the area under the growth curve and growth rate data at 72 and 96 hours for the control and all test concentrations to determine any statistically significant differences between the test and control groups. All statistical analyses were performed using the SAS computer software package (SAS 1999 - 2001).

3.3.3.5 Geometric mean measured test concentrations

The geometric mean measured test concentrations of the samples were calculated as follows using the measured test concentrations of replicates R_1 - R_3 pooled:

$$GM = \sqrt{C_0 \times C_1}$$

where

GM = geometric mean measured test concentration (mg/l)
C_0 = measured concentration at the start of the test (mg/l)
C_1 = measured concentration at the end of the test (mg/l)

3.4 Positive Control

A positive control (Safepharm Laboratories Project Number 0039/0883) used zinc chloride (Fisher Chemicals 0694439) as the reference material. An amount of reference material (50 mg) was dissolved in culture medium and the volume adjusted to 500 ml to give a 100 mg/l stock solution from which a series of dilutions were made to give a further stock solutions of 10, 2.0, 0.64, 0.20, 0.064 and 0.020 mg/l. An aliquot (250 ml) of each of the 0.020, 0.064, 0.20, 0.64 and 2.0 mg/l stock solutions was separately mixed with algal suspension (250 ml) to give the required test concentrations of 0.010, 0.032, 0.10, 0.32 and 1.0 mg/l.

The test was conducted in 250 ml glass conical flasks each containing 100 ml of test preparation and plugged with polyurethane foam bungs to reduce evaporation. Three replicate flasks were prepared for the control and each test concentration.

The flasks were incubated (INFORS Multitron® Version 2 incubator) at 24 ± 1°C under continuous illumination (intensity approximately 7000 lux) and constantly shaken at approximately 150 rpm for 72 hours.

Samples were taken at 0, 24, 48, 72 and 96 hours and the cell densities determined using a Coulter® Multisizer Particle Counter.

3.4.1 Evaluation of data for the Positive Control

3.4.1.1 Comparison of areas under the growth curves

The area under the curve is taken to be an index of growth and was calculated using the following equation:

$$A = \frac{N_1 - N_0}{2} \times t_1 + \frac{N_1 + N_2 - 2N_0}{2} \times (t_2 - t_1) + \frac{N_{n-1} + N_n - 2N_0}{2} \times (t_n - t_{n-1})$$

where

A	=	area
N_0	=	cell concentration at the start of the test
N_1	=	cell concentration at t_1
N_n	=	cell concentration at t_n
t_1	=	time of first measurement (hours from start)
t_n	=	time of n^{th} measurement (hours from start)

Percentage inhibition of growth at each test concentration (I_A) was calculated by comparing the area under the test curve (A_t) with that under the control curve (A_c) using the following equation:

$$I_A = \frac{A_c - A_t}{A_c} \times 100$$

The percentage inhibition values (I_A) (Table 6) were plotted against test concentration (Figures 12 and 13), a line fitted using the Xlfit software package (IDBS) and the EC_{50} values with respect to the area under the growth curve (biomass), E_bC_{50} (72 and 96 h) determined from the equation for the fitted line.

3.4.1.2 Comparison of growth rates

The average maximum growth rate for each culture was also calculated, from the straight section of the growth curve (Figure 11), using the following equation:

$$\mu = \frac{\ln N_n - \ln N_0}{t_n - t_0}$$

where

μ	=	average maximum growth rate
N_0	=	cell concentration at the start of the test
N_n	=	cell concentration at t_n
t_0	=	time of first measurement (0 hours)
t_n	=	time of n^{th} measurement (hours from start)

Percentage inhibition of growth rate for each test concentration (I_μ) was calculated by comparing the growth rate of the test curve (μ_t) with that of the control curve (μ_c) using the following equation:

$$I_{\mu} = \frac{\mu_c - \mu_t}{\mu_c} \times 100$$

Percentage reductions in growth rate (I_{μ}) (Table 6) were plotted against test concentration (Figure 14), a line fitted using the Xlfit software package (IDBS) and the EC_{50} value with respect to growth rate, E_rC_{50} (0 - 96 h) determined from the equation for the fitted line.

3.4.1.3 Statistical analysis

One way analysis of variance incorporating Bartlett's test for homogeneity of variance (Sokal and Rohlf 1981) and Dunnett's multiple comparison procedure for comparing several treatments with a control (Dunnett 1955) was carried out on the area under the growth curve data at 96 hours for the control and all test concentrations to determine any statistically significant differences between the test and control groups. All statistical analyses were performed using the SAS computer software package (SAS 1999 - 2001).

The 95% confidence limits were calculated using the method of Litchfield and Wilcoxon (Litchfield and Wilcoxon 1949).

4. ARCHIVES

Unless instructed otherwise by the Sponsor, all original data and the final report will be retained in the Safepharm archives for five years, after which instructions will be sought as to further retention or disposal.

5. RESULTS

5.1 Range-finding Test

The cell densities and percentage inhibition of growth values from the exposure of *Pseudokirchneriella subcapitata* to the test material during the range-finding test are given in Table 1.

The results showed no significant effect on growth at the test concentrations of 1.0 and 10 mg/l. However, growth was observed to be reduced at 100 and 1000 mg/l.

Based on this information test concentrations of 10, 32, 100, 320 and 1000 mg/l were selected for the definitive test.

5.2 Definitive Test

5.2.1 Verification of test concentrations

Chemical analysis of the test preparations at 0 hours (see Appendix 2) showed measured test concentrations to be in the range of 68% to 83% of nominal. With the exception of the 10 and 320 mg/l test concentrations which gave measured concentrations of 83% and 81% of nominal respectively the remaining test concentrations were below the normal acceptance limit of 80%.

This effect was observed in two previous tests conducted on this test material (non-reported) and hence was considered to be a true effect rather than poor preparation techniques. Examination of the recovery analysis data (see Appendix 2) indicated that in the presence of algal cells, recovery was 5 - 10% lower than when no algal cells were present. It was therefore considered that the test material showed some immediate adsorption to algal cells and that this accounted for the slightly low measured concentrations at 0 hours. This adsorption would be more pronounced in the definitive test than in the recovery analyses due to the longer time period between preparation and analysis in the definitive test.

Analysis of the test preparations at 96 hours (see Appendix 2) showed a decline in measured test concentrations with values in the range of less than the limit of quantitation (LOQ) of the analytical method, which was assessed down to 0.44 mg/l, to 49% of nominal. This decline was in line with that observed in the stability analyses conducted.

In the additional vessels prepared without the addition of algal cells and incubated alongside the definitive test vessels without being opened, whilst there was a significant decline in measured test concentrations after 96 hours, at the test concentrations of 320 and 1000 mg/l this decline was less than observed in the definitive test samples (see Appendix 2). Based on these results it could be assumed that the large losses of test material observed in the definitive test were due to a combination of instability and additional losses due to volatility when the test vessels were opened on a daily basis to remove samples for the determination of algal cell density values.

The test material was known to degrade in aqueous medium forming tert-butanol and acetic acid. Tert-butanol was detected using the analytical method employed for the parent test material, however acetic acid was not. Therefore quantitation of tert-butanol concentrations in the test samples was undertaken at 0 and 96 hours in addition to the parent test material analyses. At 0 hours measured concentrations of tert-butanol were all less than the LOQ of the analytical method which was assessed down to 3.0 mg/l (see Appendix 2). After 96 hours there was an increase in measured concentrations of the degradation product with measured concentrations in the range 4.66 to 232 mg/l. This increase in measured degradation product concentrations was in line with the decline in parent test material concentrations thereby confirming the instability of the test material under experimental conditions.

5.2.2 Growth data

From the data given in Tables 2 and 3, it is clear that both the growth (r) and the biomass (b) of *Pseudokirchneriella subcapitata* (CCAP 278/4) were affected by the presence of the test material over the 96-Hour exposure period.

The mean cell densities versus time for the definitive test are presented in Figure 1. Percentage inhibition values are plotted against nominal test concentration in Figures 2 to 5.

Effect Concentration Values for Algal Growth Inhibition (based on nominal test concentration)

Parameter (with 95% confidence limits)	72-Hour value (mg/l)	96-Hour value (mg/l)
E_rC_{10}	120	120
E_rC_{50}	530 (420 - 670)	670 (limits not calculable)
E_rC_{90}	700	>1000
E_bC_{10}	44	28
E_bC_{50}	220 (180 - 270)	210 (160 - 280)
E_bC_{90}	700	760
NOEC	32	32

where E_rC_x is the test concentration that reduced specific growth rate by x% and E_bC_x is the test concentration that reduced biomass by x%.

The following data show that the cell concentration of the control cultures increased by a factor of 92 after 72 hours and 161 after 96 hours. This increase was in line with the OECD Guideline that states the enhancement must be at least by a factor of 16 after 72 hours and the EPA Guideline that states the enhancement must be at least by a factor of 100 after 96 hours.

Mean cell density of control at 0 hours : 1.05×10^4 cells per ml
Mean cell density of control at 72 hours : 9.71×10^5 cells per ml
Mean cell density of control at 96 hours : 1.69×10^6 cells per ml

Current regulatory advice is that in cases where a decline in parent test material concentrations is observed, geometric mean measured concentrations should be used for calculating EC_{50} values. Results based on geometric mean measured test concentrations were therefore calculated in order to give a "worst case" analysis of the data. In cases where the measured concentration was less than the LOQ of the analytical method following current regulatory advice a value of half the LOQ (i.e. 0.22 mg/l) was used to enable calculation of the geometric mean measured concentration. The geometric mean measured test concentrations were determined to be:

Nominal Test Concentration (mg/l)	Geometric Mean Measured Test Concentration (mg/l)	Expressed as a % of the Nominal Test Concentration
10	1.8	18
32	2.3	7
100	4.1	4
320	7.5	2
1000	573	57

The following results were determined from the data based on the geometric mean measured test concentrations:

Effect Concentration Values for Algal Growth Inhibition (based on geometric mean measured test concentrations)

Parameter (with 95% confidence limits)	72-Hour value (mg/l)	96-Hour value (mg/l)
E_rC_{10}	4.3	3.7
E_rC_{50}	16 (11 - 23)	64 (limits not calculable)
E_rC_{90}	130	>570
E_bC_{10}	2.8	2.3
E_bC_{50}	6.1 (5.4 - 6.9)	5.8 (4.9 - 6.9)
E_bC_{90}	15	23
NOEC	2.3	2.3

The use of the geometric mean measured test concentrations in the calculation of the EC_{50} and NOEC values had a significant effect on the outcome of the study. This was due to the unstable nature of the test material in aqueous medium over the test period.

5.2.3 Observations on cultures

All test and control cultures were inspected microscopically at 96 hours. After 96 hours there were no abnormalities detected in the control or test cultures at 10, 32, 100 and 320 mg/l, however cell debris was observed to be present in the test cultures at 1000 mg/l.

5.2.4 Observations on test material solubility

At 0 hours all control and test cultures were clear colourless solutions. After the 96-Hour exposure period the control, 10, 32 and 100 mg/l test cultures were bright green dispersions and the 320 mg/l test cultures were green dispersions. The 1000 mg/l test cultures remained as clear colourless solutions throughout the test period.

5.2.5 Physico-chemical measurements

The pH values of each test and control flask are given in Table 2. Temperature was maintained at 24 ± 1°C throughout the test.

The pH values of the control cultures (see Table 2) were observed to increase from pH 7.7 at 0 hours to pH 11.0 - 11.2 at 96 hours. This increase was considered to be due to the amount of carbon dioxide required by the large number of algal cells in the log phase of growth (see Figure 1) exceeding the transfer rate of CO_2 from the gaseous phase to the aqueous phase. In this situation CO_2 required for photosynthesis and growth would be derived from bicarbonate in solution which results in an increase in the pH of the culture and could not be avoided due to the requirement to conduct the test in sealed vessels to minimise losses of test material due to volatility. The increase in pH was in excess of that recommended in the Test Guidelines (1.5 pH units after 72 hours). This was considered to have had no adverse effect on the results of the study given that the increase in cell concentration in the control cultures exceeded the validation criterion given in the Test Guidelines.

5.2.6 Re-growth experiment

A re-growth experiment was performed after 96 hours exposure to determine the algicidal or algistatic effect of the test material. Aliquots (0.5 ml) were removed for each replicate culture and the replicates pooled for each concentration. Fresh sterile culture medium (100 ml) was added to

ensure that the test concentrations were reduced to below the inhibiting level. The sub-cultures were incubated at 24 ± 1°C for a period of 120 hours. Re-growth occurred in the control, 10 and 32 mg/l test cultures after 48 hours, in the 100 mg/l test culture after 72 hours, in the 320 mg/l test culture after 96 hours and in the 1000 mg/l test culture after 120 hours. These results indicated that the test material may have been algistatic rather than algicidal in effect.

5.3 Positive Control

The cell densities from exposure of *Pseudokirchneriella subcapitata* (CCAP 278/4) to the reference material are given in Table 5 and Figure 11 and the inhibition of growth values are given in Table 6 and Figures 12, 13 and 14.

Accordingly the following results were determined from the data:

E_bC_{50} (72 h) : 0.14 mg/l; 95% confidence limits 0.12 - 0.16 mg/l
E_bC_{50} (96 h) : 0.17 mg/l *
E_rC_{50} (0 - 96 h) : 0.39 mg/l *
No Observed Effect Concentration (NOEC): 0.10 mg/l.

The mean E_bC_{50} (96 h) value calculated from all positive controls is 0.12 mg/l (sd = 0.038) and the mean E_rC_{50} (0 – 96 h) value is 0.42 mg/l (sd = 0.13).

6. CONCLUSION

The effect of the test material on the growth of *Pseudokirchneriella subcapitata* has been investigated over a 96-Hour period and gave an E_bC_{50} (72 h) value of 220 mg/l; 95% confidence limits 180 - 270 mg/l and an E_bC_{50} (96 h) value of 210 mg/l, 95% confidence limits 160 - 280 mg/l based on nominal concentrations. The E_rC_{50} (0 - 72 h) value based on nominal test concentrations was 530 mg/l*; 95% confidence limits 420 - 670 mg/l and the E_rC_{50} (0 - 96 h) value was 670 mg/l. The No Observed Effect Concentration at 72 and 96 hours was 32 mg/l.

Based on the geometric mean measured test concentrations the E_bC_{50} (72 h) value was 6.1 mg/l; 95% confidence limits 5.4 - 6.9 mg/l, the E_bC_{50} (96 h) value was 5.8 mg/l, 95% confidence limits 4.9 - 6.9 mg/l, the E_rC_{50} (0 - 72 h) value was 16 mg/l; 95% confidence limits 11 - 23 mg/l, the E_rC_{50} (0 - 96 h) value was 64 mg/l and the No Observed Effect Concentration at 96 hours was 2.3 mg/l.

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* It was not possible to calculate 95% confidence limits for these EC_{50} values as the data generated did not fit the models available for the calculation of confidence limits.

7. REFERENCES

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tert-Butyl acetate: ALGAL INHIBITION TEST

Table 1 Cell Densities and Percentage Inhibition of Growth from the Range-finding Test

Nominal Concentration (mg/l)		Cell Densities* (cells per ml)			
		0 Hours	96 Hours	% Inhibition (area under curve at 96 h)	% Inhibition (growth rate 0 – 96 h)
Control	R_1	1.07E+04	1.53E+06		
	R_2	1.04E+04	1.56E+06	-	-
	Mean	1.06E+04	1.55E+06		
1.0	R_1	1.02E+04	1.98E+06		
	R_2	9.77E+03	2.38E+06	[42]	[8]
	Mean	9.99E+03	2.18E+06		
10	R_1	1.07E+04	1.88E+06		
	R_2	1.09E+04	1.78E+06	[19]	[2]
	Mean	1.08E+04	1.83E+06		
100	R_1	1.11E+04	8.35E+05		
	R_2	1.08E+04	8.52E+05	46	13
	Mean	1.09E+04	8.43E+05		
1000	R_1	1.16E+04	1.29E+04		
	R_2	9.50E+03	1.39E+04	100	96
	Mean	1.05E+04	1.34E+04		

* Cell densities represent the mean number of cells per ml calculated from the mean of the cell counts from 3 counts for each of the replicate flasks.

R_1 and R_2 = Replicates 1 and 2

[Increase in growth compared to controls]

tert-Butyl acetate: ALGAL INHIBITION TEST

Table 2 Cell Densities and pH Values in the Definitive Test

Nominal Concentration (mg/l)		pH	Cell Densities* (cells per ml)					pH
		0 h	0 h	24 h	48 h	72 h	96 h	96 h
Control	R_1	7.7	1.02E+04	8.49E+04	4.31E+05	9.11E+05	1.85E+06	11.0
	R_2	7.7	1.08E+04	8.20E+04	3.70E+05	1.09E+06	1.50E+06	11.2
	R_3	7.7	1.04E+04	8.72E+04	4.04E+05	9.12E+05	1.72E+06	11.2
	Mean		1.05E+04	8.47E+04	4.02E+05	9.71E+05	1.69E+06	
10	R_1	7.8	1.11E+04	8.27E+04	4.04E+05	8.39E+05	1.66E+06	10.9
	R_2	7.8	1.36E+04	8.64E+04	4.57E+05	1.10E+06	1.62E+06	10.7
	R_3	7.8	9.98E+03	8.30E+04	4.13E+05	9.21E+05	1.58E+06	10.8
	Mean		1.16E+04	8.40E+04	4.24E+05	9.52E+05	1.62E+06	
32	R_1	7.8	1.07E+04	7.95E+04	3.91E+05	1.01E+06	1.58E+06	10.5
	R_2	7.8	1.07E+04	7.16E+04	3.79E+05	9.04E+05	1.25E+06	10.4
	R_3	7.8	1.01E+04	8.55E+04	3.32E+05	9.11E+05	1.41E+06	10.5
	Mean		1.05E+04	7.89E+04	3.67E+05	9.43E+05	1.42E+06	
100	R_1	7.8	9.56E+03	7.28E+04	3.11E+05	7.00E+05	1.11E+06	10.0
	R_2	7.8	1.10E+04	6.89E+04	3.10E+05	6.18E+05	8.27E+05	9.8
	R_3	7.8	9.99E+03	7.55E+04	3.66E+05	6.15E+05	9.23E+05	9.9
	Mean		1.02E+04	7.24E+04	3.29E+05	6.44E+05	9.52E+05	
320	R_1	7.8	1.07E+04	4.50E+04	1.58E+05	3.38E+05	6.93E+05	8.9
	R_2	7.8	1.38E+04	4.24E+04	1.61E+05	3.75E+05	7.32E+05	8.7
	R_3	7.8	1.18E+04	3.95E+04	1.58E+05	3.40E+05	7.50E+05	8.6
	Mean		1.21E+04	4.23E+04	1.59E+05	3.51E+05	7.25E+05	
1000	R_1	7.8	1.03E+04	1.56E+04	6.50E+03	1.28E+04	4.25E+04	7.5
	R_2	7.8	1.07E+04	1.54E+04	6.27E+03	1.18E+04	2.78E+04	7.6
	R_3	7.8	1.07E+04	1.63E+04	7.58E+03	1.25E+04	4.39E+04	7.5
	Mean		1.06E+04	1.57E+04	6.78E+03	1.24E+04	3.81E+04	

* Cell densities represent the mean number of cells per ml calculated from the mean of the cell counts from 3 counts for each of the replicate flasks.

R_1 - R_3 = Replicates 1 to 3

tert-Butyl acetate: ALGAL INHIBITION TEST

Table 3 Inhibition of Growth Rate and Biomass After 72 Hours

Nominal Concentration (mg/l)	Area Under Curve at 72 h	% Inhibition	Growth Rate (0 - 72h)	% Inhibition
Control	2.27E+07	-	0.063	-
10	2.29E+07	[1]	0.061	3
32	2.14E+07	6	0.062	2
100	1.68E+07	26	0.058	8
320	8.31E+06	63	0.047	25
1000	5.48E+04	100	0.002	97

[Increase in growth compared to controls]

tert-Butyl acetate: ALGAL INHIBITION TEST

Table 4 Inhibition of Growth Rate and Biomass After 96 Hours

Nominal Concentration (mg/l)	Area Under Curve at 96 h	% Inhibition	Growth Rate (0 - 96h)	% Inhibition
Control	5.44E+07	-	0.053	-
10	5.35E+07	2	0.052	2
32	4.94E+07	9	0.051	4
100	3.57E+07	34	0.047	11
320	2.09E+07	61	0.042	21
1000	4.07E+05	99	0.013	75

tert-Butyl acetate: ALGAL INHIBITION TEST

Table 5 Cell Densities and pH Values in the Positive Control

Nominal Concentration (mg/l)		pH	Cell Densities* (cells per ml)					pH
		0 h	0 h	24 h	48 h	72 h	96 h	96 h
Control	R_1	7.4	1.18E+04	2.39E+04	1.13E+05	8.94E+05	1.23E+06	7.7
	R_2	7.4	1.05E+04	2.48E+04	1.14E+05	9.06E+05	1.21E+06	7.7
	R_3	7.4	1.02E+04	2.30E+04	1.39E+05	8.81E+05	1.19E+06	7.7
	Mean		1.08E+04	2.39E+04	1.22E+05	8.94E+05	1.21E+06	
0.010	R_1	7.4	9.62E+03	2.53E+04	1.34E+05	9.84E+05	1.31E+06	7.7
	R_2	7.4	1.03E+04	2.56E+04	1.47E+05	9.86E+05	1.28E+06	7.8
	R_3	7.4	9.84E+03	2.56E+04	1.42E+05	1.02E+06	1.29E+06	7.8
	Mean		9.92E+03	2.55E+04	1.41E+05	9.96E+05	1.29E+06	
0.032	R_1	7.4	1.08E+04	2.76E+04	1.39E+05	1.02E+06	1.48E+06	7.7
	R_2	7.4	9.77E+03	2.51E+04	1.43E+05	9.51E+05	1.41E+06	7.7
	R_3	7.4	9.47E+03	2.41E+04	1.35E+05	1.13E+06	1.59E+06	7.7
	Mean		1.00E+04	2.56E+04	1.39E+05	1.03E+06	1.49E+06	
0.10	R_1	7.4	9.72E+03	2.34E+04	1.06E+05	6.84E+05	1.56E+06	7.6
	R_2	7.4	9.68E+03	2.42E+04	9.86E+04	6.55E+05	1.56E+06	7.5
	R_3	7.4	9.36E+03	2.36E+04	1.10E+05	6.86E+05	1.77E+06	7.6
	Mean		9.59E+03	2.37E+04	1.05E+05	6.75E+05	1.63E+06	
0.32	R_1	7.3	9.80E+03	1.25E+04	1.86E+04	8.16E+04	1.38E+05	7.1
	R_2	7.3	9.45E+03	1.26E+04	1.78E+04	5.24E+04	8.37E+04	7.1
	R_3	7.3	9.07E+03	1.34E+04	1.63E+04	5.63E+04	1.04E+05	7.1
	Mean		9.44E+03	1.28E+04	1.75E+04	6.34E+04	1.08E+05	
1.0	R_1	7.1	9.38E+03	1.05E+04	1.25E+04	2.29E+04	4.66E+04	7.0
	R_2	7.1	9.74E+03	1.04E+04	9.46E+03	1.72E+04	4.02E+04	7.0
	R_3	7.1	9.25E+03	1.04E+04	1.03E+04	1.46E+04	1.78E+04	7.0
	Mean		9.46E+03	1.04E+04	1.07E+04	1.82E+04	3.49E+04	

* Cell densities represent the mean number of cells per ml calculated from the mean of the cell counts from 3 counts for each of the replicate flasks.

R_1 - R_3 = Replicates 1 to 3

tert-Butyl acetate: ALGAL INHIBITION TEST

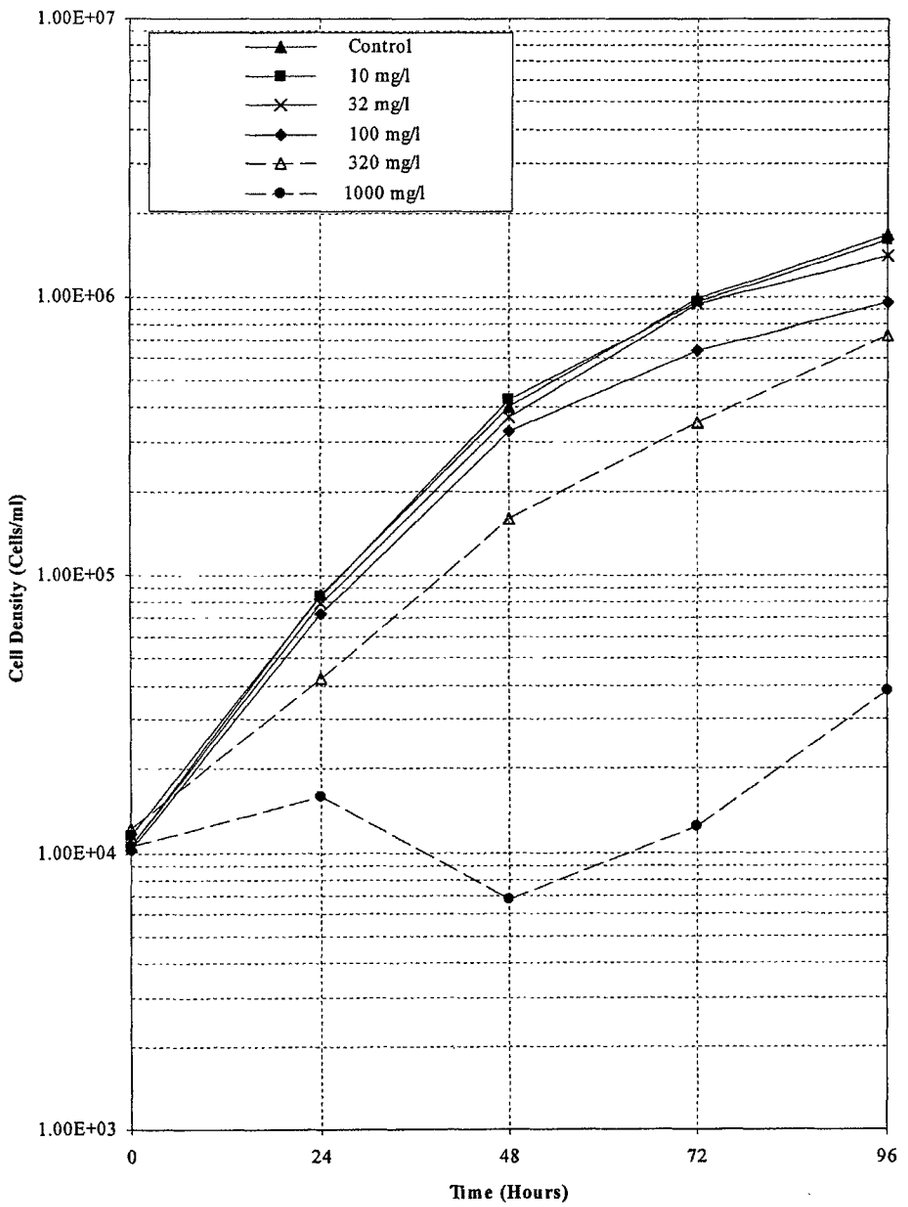
Table 6 Inhibition of Growth Rate and Biomass in the Positive Control

Nominal Concentration (mg/l)	Area Under Curve at 72 h	% Inhibition	Area Under Curve at 96 h	% Inhibition	Growth Rate (0 – 96 h)	% Inhibition
Control	1.36E+07	-	3.85E+07	-	0.049	-
0.010	1.54E+07	[13]	4.26E+07	[11]	0.051	[4]
0.032	1.58E+07	[16]	4.59E+07	[19]	0.052	[6]
0.10	1.06E+07	22	3.81E+07	1	0.054	[10]
0.32	9.24E+05	93	2.76E+06	93	0.025	49
1.0	1.60E+05	99	5.70E+05	99	0.013	73

[Increase in growth as compared to the control]

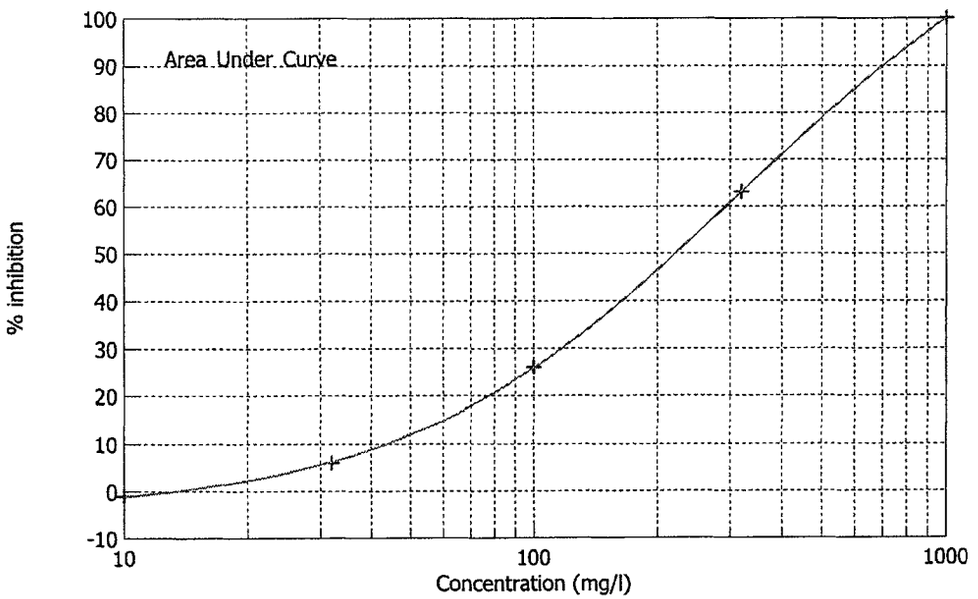
tert-Butyl acetate: ALGAL INHIBITION TEST

Figure 1 Mean Cell Densities v Time for the Definitive Test



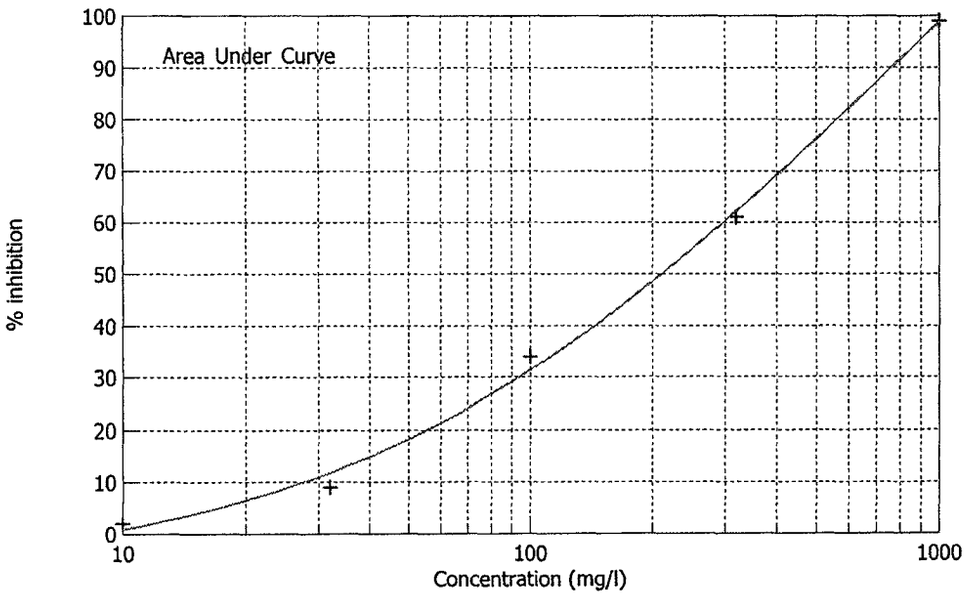
tert-Butyl acetate: ALGAL INHIBITION TEST

Figure 2 Inhibition of Biomass at 72 Hours Based on Nominal Test Concentrations



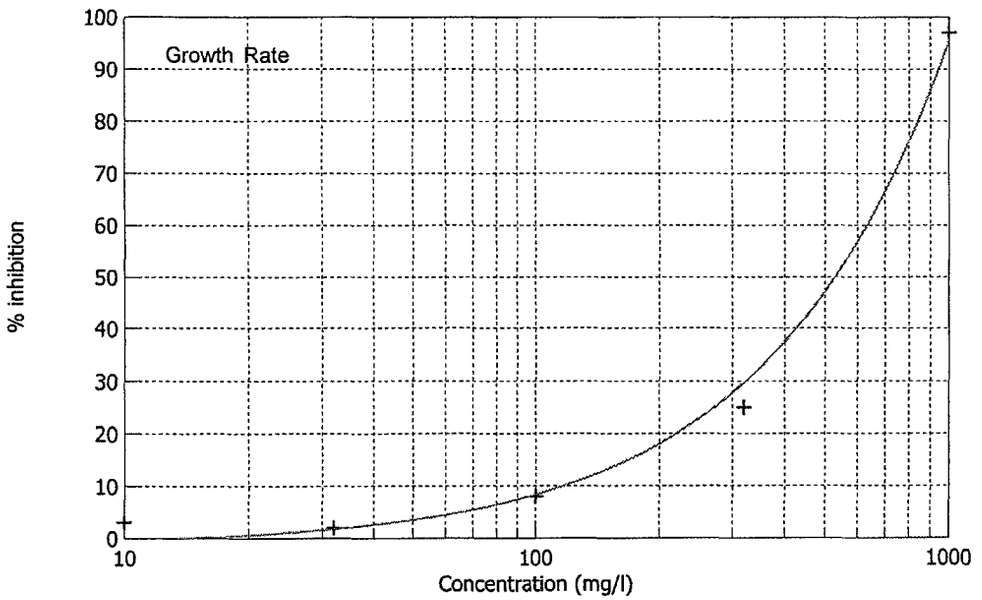
tert-Butyl acetate: ALGAL INHIBITION TEST

Figure 3 Inhibition of Biomass at 96 Hours Based on Nominal Test Concentrations



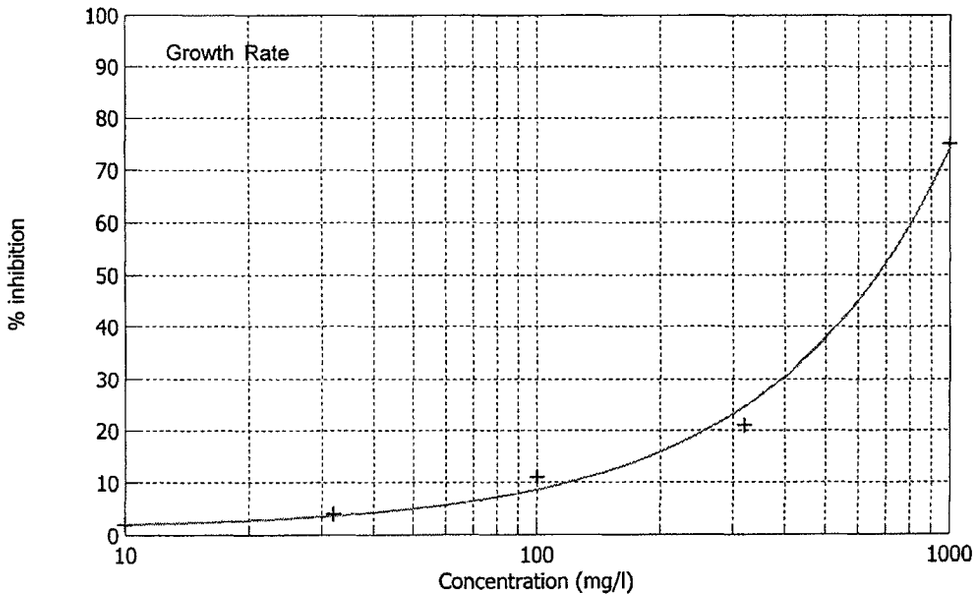
tert-Butyl acetate: ALGAL INHIBITION TEST

Figure 4 **Inhibition of Growth Rate (0 – 72 hours) Based on Nominal Test Concentrations**



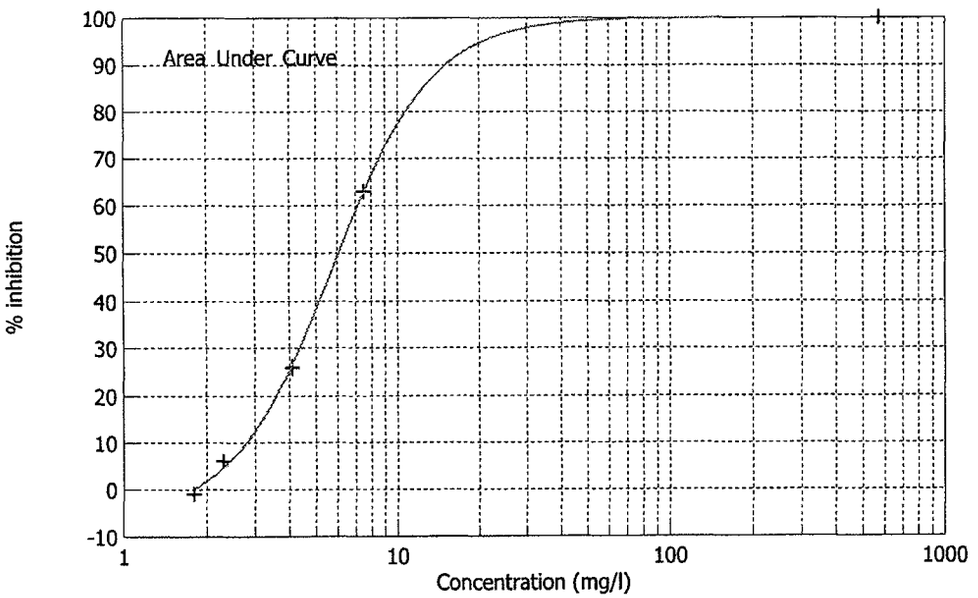
tert-Butyl acetate: ALGAL INHIBITION TEST

Figure 5 **Inhibition of Growth Rate (0 – 96 hours) Based on Nominal Test Concentrations**



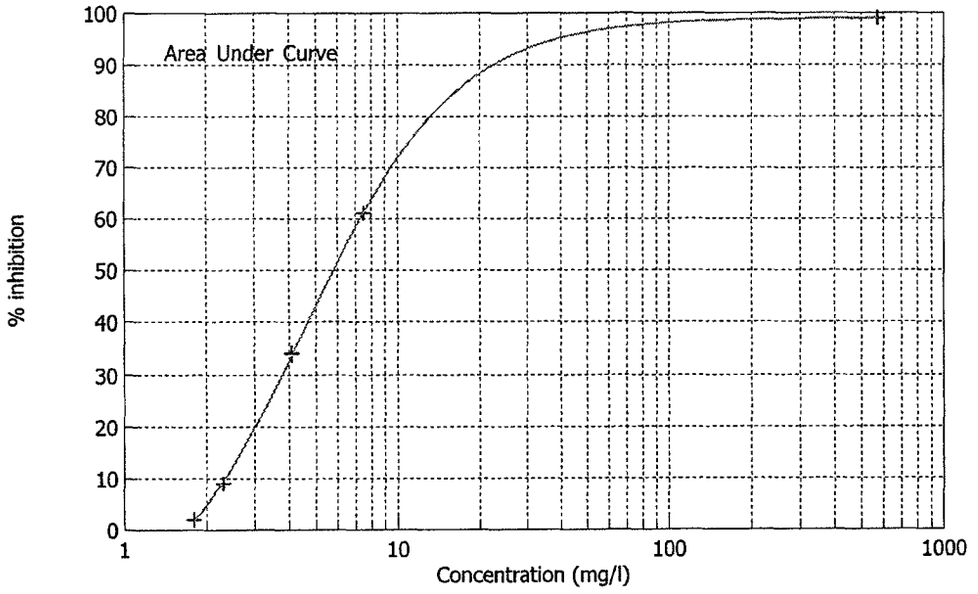
tert-Butyl acetate: ALGAL INHIBITION TEST

Figure 6 **Inhibition of Biomass at 72 Hours Based on Geometric Mean Measured Test Concentrations**



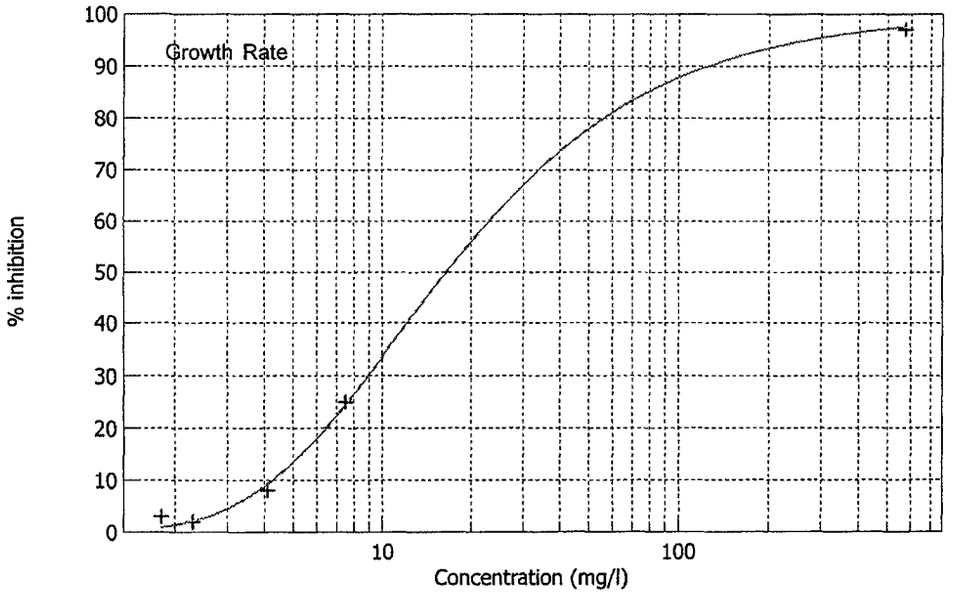
tert-Butyl acetate: ALGAL INHIBITION TEST

Figure 7 Inhibition of Biomass at 96 Hours Based on Geometric Mean Measured Test Concentrations



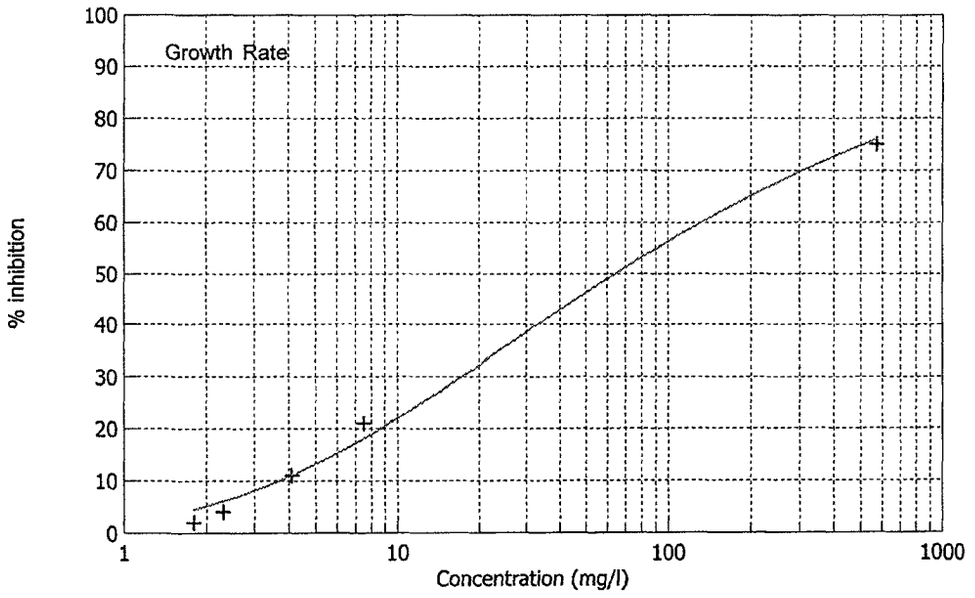
tert-Butyl acetate: ALGAL INHIBITION TEST

Figure 8 **Inhibition of Growth Rate (0 – 72 hours) Based on Geometric Mean Measured Test Concentrations**



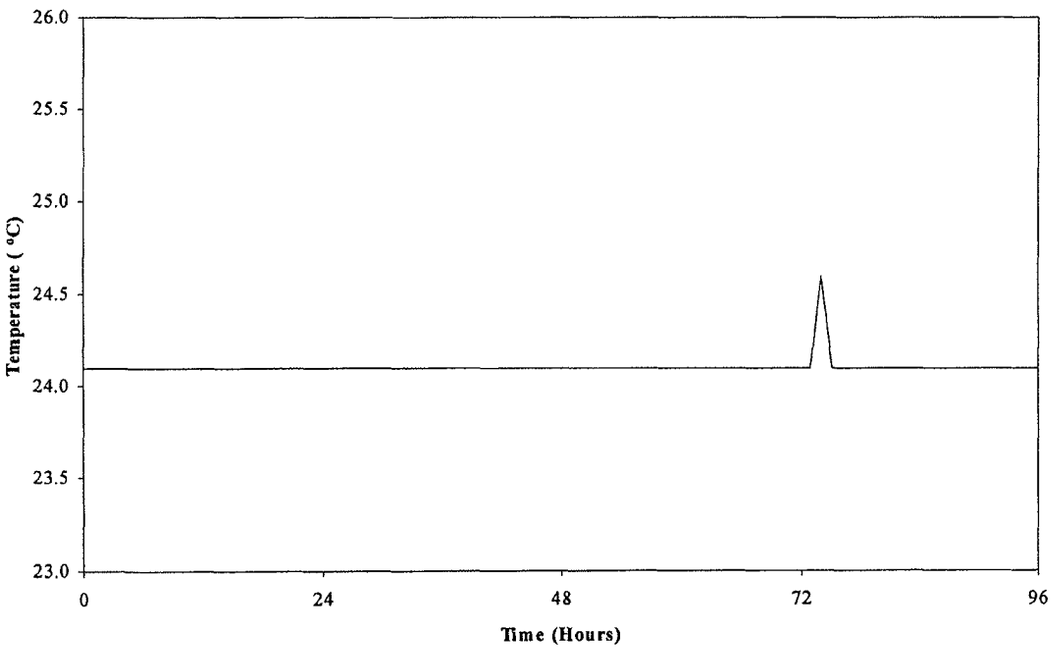
tert-Butyl acetate: ALGAL INHIBITION TEST

Figure 9 **Inhibition of Growth Rate (0 – 96 hours) Based on Geometric Mean Measured Test Concentrations**



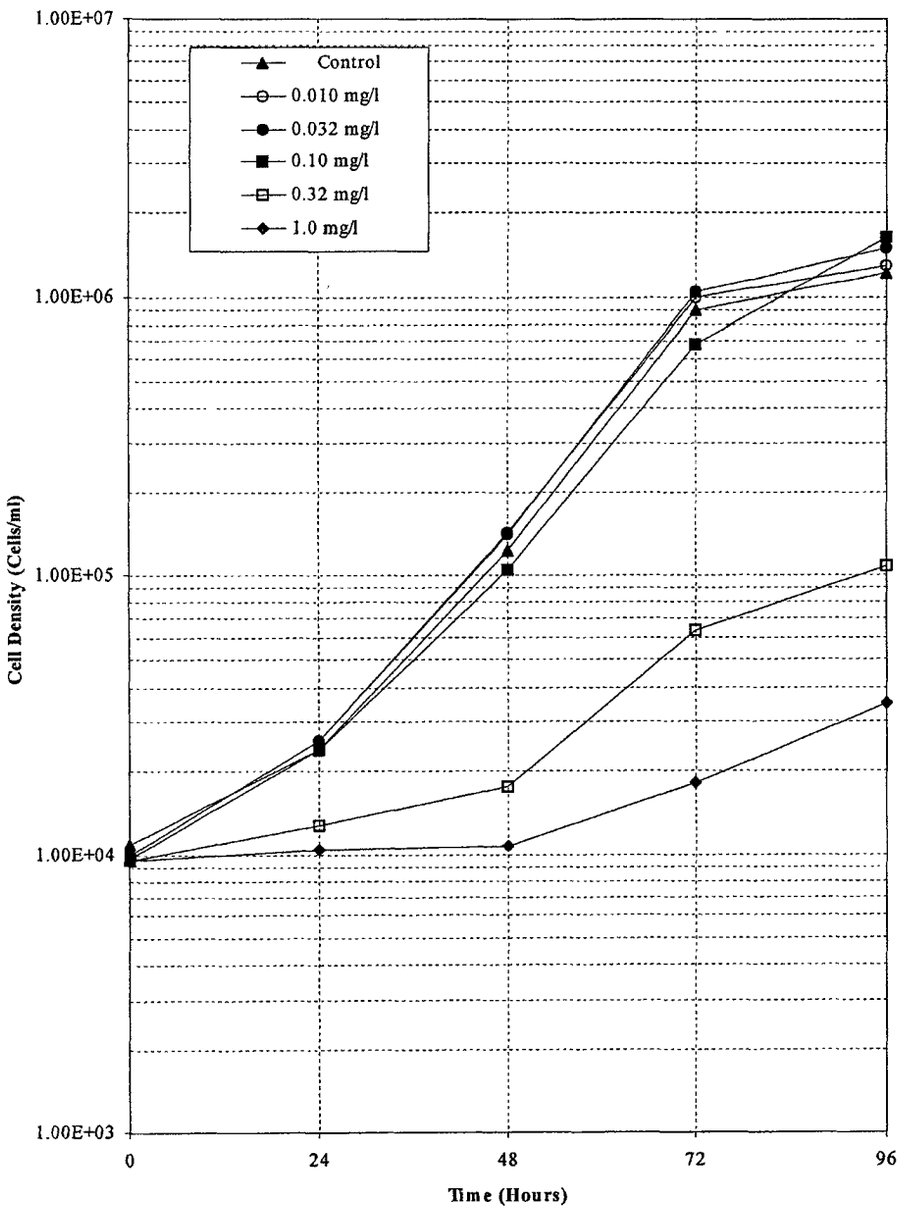
tert-Butyl acetate: ALGAL INHIBITION TEST

Figure 10 Temperature Measurements Taken Throughout the Test Period



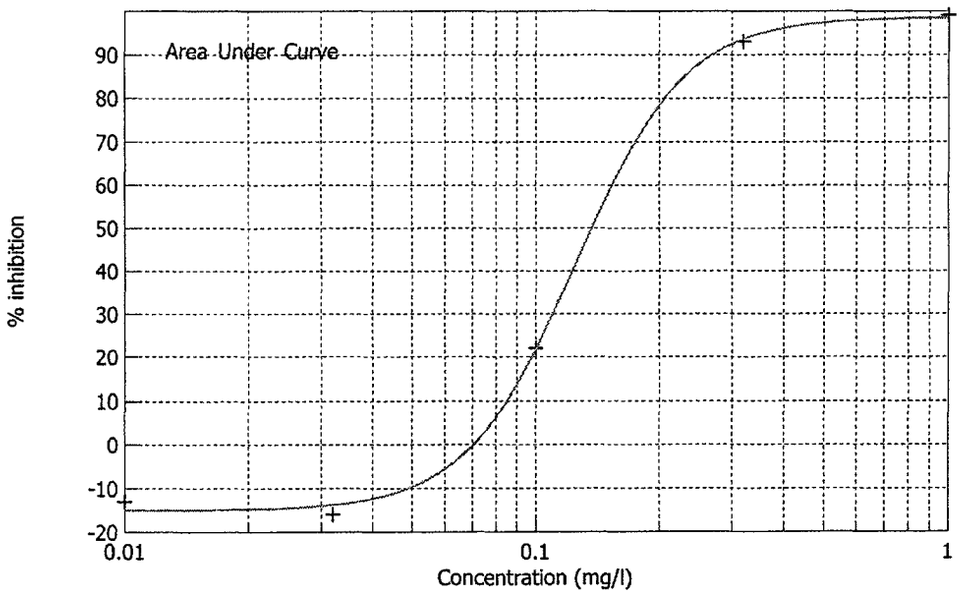
tert-Butyl acetate: ALGAL INHIBITION TEST

Figure 11 Mean Cell Densities v Time for the Positive Control



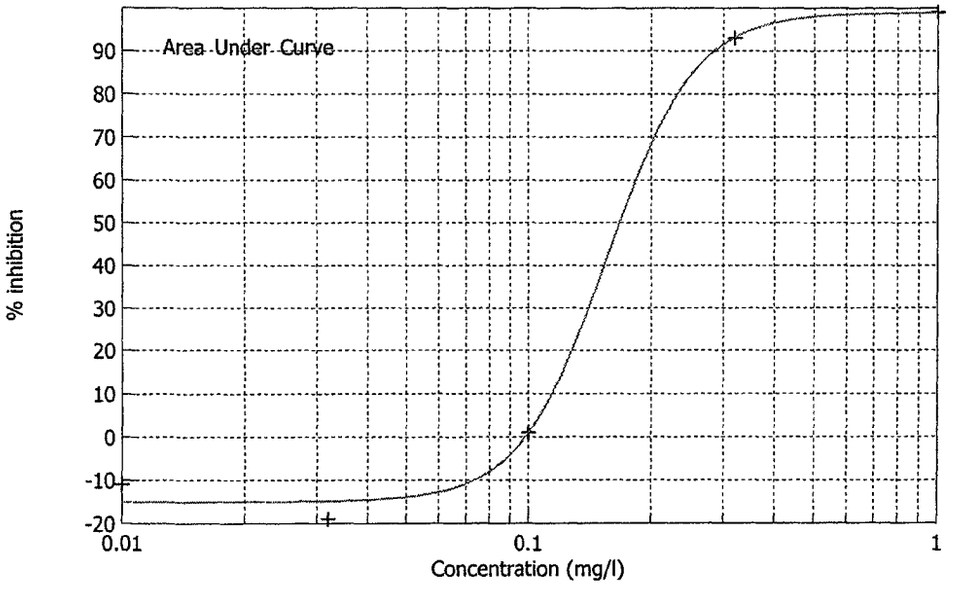
tert-Butyl acetate: ALGAL INHIBITION TEST

Figure 12 Inhibition of Biomass at 72 Hours for the Positive Control



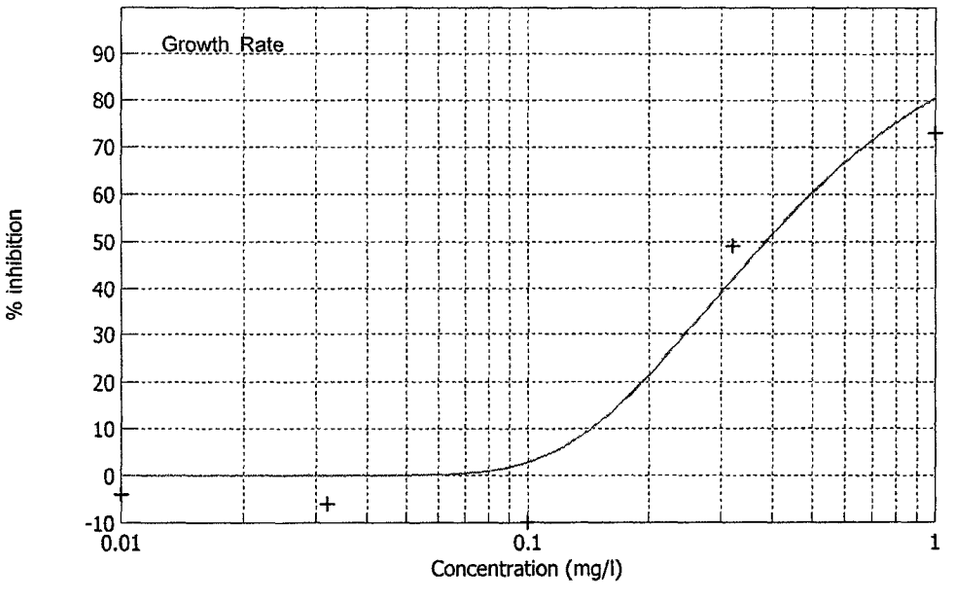
tert-Butyl acetate: ALGAL INHIBITION TEST

Figure 13 Inhibition of Biomass at 96 Hours for the Positive Control



tert-Butyl acetate: ALGAL INHIBITION TEST

Figure 14 Inhibition of Growth Rate (0 – 96 hours) for the Positive Control



Appendix 1 Culture Medium

$NaNO_3$	25.5	mg/l
$MgCl_2.6H_2O$	12.164	mg/l
$CaCl_2.2H_2O$	4.41	mg/l
$MgSO_4.7H_2O$	14.7	mg/l
K_2HPO_4	1.044	mg/l
$NaHCO_3$	15.0	mg/l
H_3BO_3	0.1855	mg/l
$MnCl_2.4H_2O$	0.415	mg/l
$ZnCl_2$	0.00327	mg/l
$FeCl_3.6H_2O$	0.159	mg/l
$CoCl_2.6H_2O$	0.00143	mg/l
$Na_2MoO_4.2H_2O$	0.00726	mg/l
$CuCl_2.2H_2O$	0.000012	mg/l
$Na_2EDTA.2H_2O$	0.30	mg/l
$Na_2SeO_3.5H_2O$	0.000010	mg/l

The culture medium was prepared using reverse osmosis purified deionised water* and the pH adjusted to 7.5 ± 0.1 with 0.1N NaOH or HCl.

For the purposes of the range-finding and definitive test, additional sodium bicarbonate (500 mg/l) was added to the prepared culture medium prior to use.

* Elga Optima 15+ or Elga Purelab Option R-15 BP

Appendix 2 Verification of Test Concentrations

1. METHOD OF ANALYSIS

1.1 Introduction

The test material concentration in the test samples was determined by gas chromatography (GC) using an external standard. The test material gave a chromatographic profile consisting of a single peak.

The method was developed by the Department of Analytical Services, Safepharm Laboratories Limited.

The test material was known to degrade to give tert-butanol and acetic acid. Test samples were monitored for the test material and for the degradation product, tert-butanol only.

1.2 Sample Preparation

1.2.1 Initial Validation

A volume of test sample was diluted with acetone to give a final theoretical concentration of 5.0 to 500 mg/l.

1.2.2 Definitive Test

A volume of test sample was diluted with acetone to give a final theoretical concentration of 2.5 to 250 mg/l.

1.3 Standards

1.3.1 Initial Validation

Standard solutions of test material were prepared in acetone:water* 50:50 v/v at a nominal concentration of 50 mg/l.

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* Prepared by ELGA Purelab Option R-15 BP water purification.

Appendix 2 (continued) Verification of Test Concentrations

1.3.2 Definitive Test

Standard solutions of test material and tert-butanol* were prepared in acetone:water** 75:25 v/v at a nominal concentration of 50 mg/l.

1.4 Procedure

The standards and samples were analysed by GC using the following conditions:

GC System	:	Agilent Technologies 5890 incorporating autosampler and workstation
Column	:	ZB-624 (30 m x 0.32 mm id, 1.8 µm film)
Oven temperature program	:	initial 30°C for 1 minute rate 10°C/minute final 150°C for 1 minute
Injector temperature	:	100°C
Detector temperature	:	150°C
Carrier gas and pressure	:	nitrogen at 10 psi
Injection volume	:	1 µl
Injection mode	:	splitless purge on at 1 minute
Retention times	:	approximately 7 minutes for test material approximately 4 minutes for degradant

2. VALIDATION

2.1 Test Material

2.1.1 Linearity

A range of standard solutions covering 1.1 to 1050 mg/l (44% of the lowest working concentration to 420% of the highest working concentration) was analysed.

Linearity was confirmed (R^2 =1) in the range 0 to 1050 mg/l.

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* ACROS Organics, purity ≥ 99.4%, product code 10771 0010.
** Prepared by ELGA Purelab Option R-15 BP water purification.

Appendix 2 (continued) Verification of Test Concentrations

The results are presented graphically on page 47.

2.1.2 Recoveries

2.1.2.1 Initial Validation

A range of preliminary test samples, accurately fortified at known concentrations of test material, was prepared and analysed.

The recovery samples were prepared by direct addition of the test material to a sample of test medium.

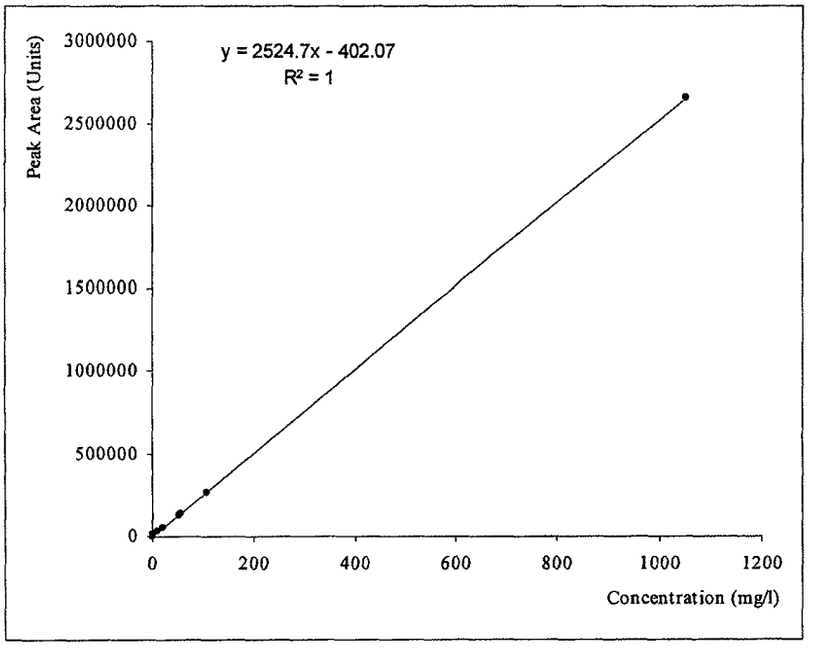
Further portions of test samples were analysed following the addition of algal cells to assess the effects of algae on the recovery of test material from test media.

Fortification (mg/l)	Recoveries		
	(mg/l)	(%)	Mean %
10.1	9.39	93	92
10.1	9.24	91	
10.1 plus algae	8.84	87	-
101	90.7	89	90
101	91.4	90	
1010	967	95	98
1010	1014	100	
1010 plus algae	881	87	-

tert-Butyl acetate: ALGAL INHIBITION TEST

Appendix 2 (continued) Verification of Test Concentrations

Linearity of Detector Response for Test Material



Appendix 2 (continued) Verification of Test Concentrations

2.1.2.2 Definitive Test

A range of preliminary test samples, accurately fortified at known concentrations of test material, was prepared and analysed.

The recovery samples were prepared by direct addition of the test material to a sample of test medium.

Further portions of test samples were analysed following the addition of algal cells to assess the effects of algae on the recovery of test material from test media.

Fortification (mg/l)	Recoveries		
	(mg/l)	(%)	Mean %
11.1	11.2	101	101
11.1	11.3	101	
11.1 plus algae	10.1	91	-
111	107	96	97
111	107	97	
1110	1060	95	96
1110	1070	97	
1110 plus algae	882	79	-

The method has been considered to be sufficiently accurate and precise for the purposes of this test. All test sample results have not been corrected for recovery.

The presence of algal cells was considered to have a slight effect on the recovery of the test material from the medium; this gave a recovery of approximately 5 to 10% lower than in the samples with no algal cells added.

2.1.3 Limit of Quantitation

The limit of quantitation has been assessed down to 0.44 mg/l.

Appendix 2 (continued) Verification of Test Concentrations

2.2 Degradation Product

2.2.1 Linearity

A range of standard solutions covering 1.5 to 230 mg/l (3% to 460% of the standard concentration) was analysed.

Linearity was confirmed (R^2 =0.9994) in the range 0 to 230 mg/l.

The results are presented graphically on page 50.

2.2.2 Recoveries

A range of preliminary test samples, accurately fortified at known concentrations of degradation product, was prepared and analysed.

The recovery samples were prepared by direct addition of the degradation product to a sample of test medium.

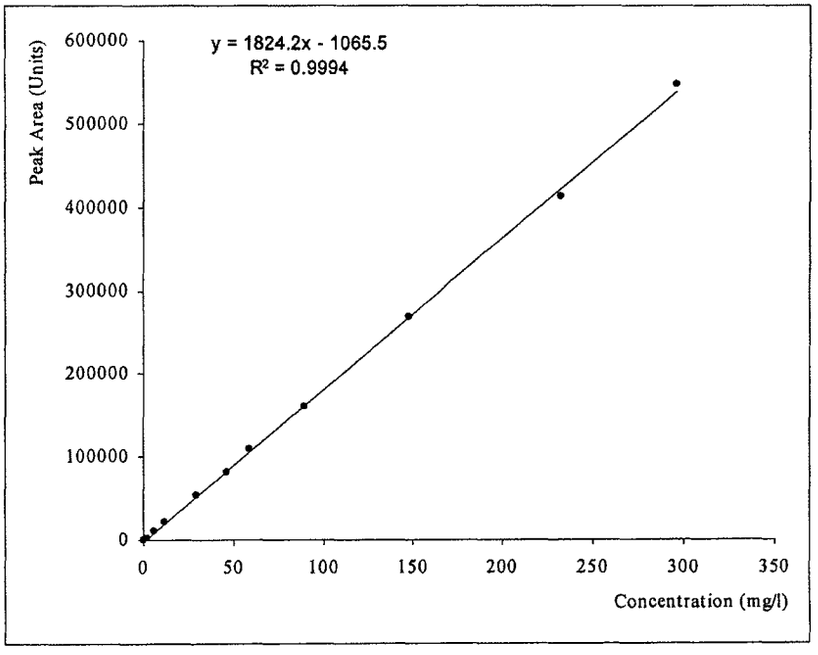
Fortification (mg/l)	Recoveries	
	(mg/l)	(%)
12.0	12.4	104
120	119	100
1200	1220	102

The method has been considered to be sufficiently accurate for the purposes of this test. All test sample results have not been corrected for recovery.

tert-Butyl acetate: ALGAL INHIBITION TEST

Appendix 2 (continued) Verification of Test Concentrations

Linearity of Detector Response for Degradation Product



Appendix 2 (continued) Verification of Test Concentrations

2.2.3 Limit of Quantitation

The limit of quantitation has been assessed down to 3.0 mg/l.

3. STABILITY

A range of preliminary test samples was prepared, analysed initially and then after storage in sealed glass vessels at ambient temperature in light and dark conditions for approximately 96 hours (equivalent to the test exposure period). In addition test samples were tested for stability without prior mixing (sonication) the test sample bottles to assess for losses due to adsorption and/or insolubility. In addition test samples were tested for stability after storage in open glass vessels in dark conditions to assess for losses due to volatility.

Stability analysis was performed on samples of test material only, not on samples of degradation product.

Nominal concentration (mg/l)	10	100	1000
Concentration found initially (mg/l)	11.2	107	1070
Concentration found after storage in light conditions (mg/l)	<LOQ	30.3	1000
Expressed as a percent of the initial concentration	-	28	94
Concentration found after storage in dark conditions (mg/l)	<LOQ	25.4	999
Expressed as a percent of the initial concentration	-	24	94
Concentration found after storage in dark conditions (mg/l) – unsonicated sample	<LOQ	NA	1010
Expressed as a percent of the initial concentration	-	-	95
Concentration found after storage in dark conditions (mg/l) – open vessel sample	<LOQ	NA	<LOQ
Expressed as a percent of the initial concentration	-	-	-

The test samples have been shown to be unstable in the test medium in light and dark conditions with the exception of the highest level. However, it can be expected that with an increase in pH

LOQ = Limit of quantitation
NA = Not Applicable

Appendix 2 (continued) Verification of Test Concentrations

(more alkaline), an increase in the rate of hydrolysis is likely. This was observed in the definitive test samples, as the presence of algal cells generally increases the pH towards the alkaline side.

The results of the unsonicated stability vessels showed no evidence of insolubility or adherence to glass.

The results of the open vessels showed evidence of volatility.

4. RESULTS

4.1 Test Material

Sample	Nominal Concentration (mg/l)	Concentration Found (mg/l)	Expressed as a Percent of the Nominal Concentration (%)
0 Hours	Control	<LOQ	-
	10	8.35	83
	32	24.9	78
	100	77.1	77
	320	259	81
	1000	675	68
96 Hours	Control	<LOQ	-
	10	<LOQ	-
	32	<LOQ	-
	100	<LOQ	-
	320	<LOQ	-
	1000	487	49
96 Hours Unopened	Control	<LOQ	-
	10	<LOQ	-
	32	<LOQ	-
	100	<LOQ	-
	320	43.4	14
	1000	763	76

LOQ = Limit of quantitation

Appendix 2 (continued) Verification of Test Concentrations

4.2 t-Butyl alcohol (hydrolysis product)

Sample	Nominal t-Butyl acetate Concentration (mg/l)	Concentration Found (mg/l)	Expressed as a Percent of the Nominal Concentration* (%)
0 Hours	Control	<LOQ	-
	10	<LOQ	-
	32	<LOQ	-
	100	<LOQ	-
	320	<LOQ	-
	1000	<LOQ	-
96 Hours	Control	<LOQ	-
	10	4.66	74
	32	15.9	78
	100	50.3	79
	320	170	83
	1000	232	36
96 Hours	Control	<LOQ	-
Unopened	10	4.11	64
	32	16.1	78
	100	49.7	78
	320	137	67
	1000	45.5	8

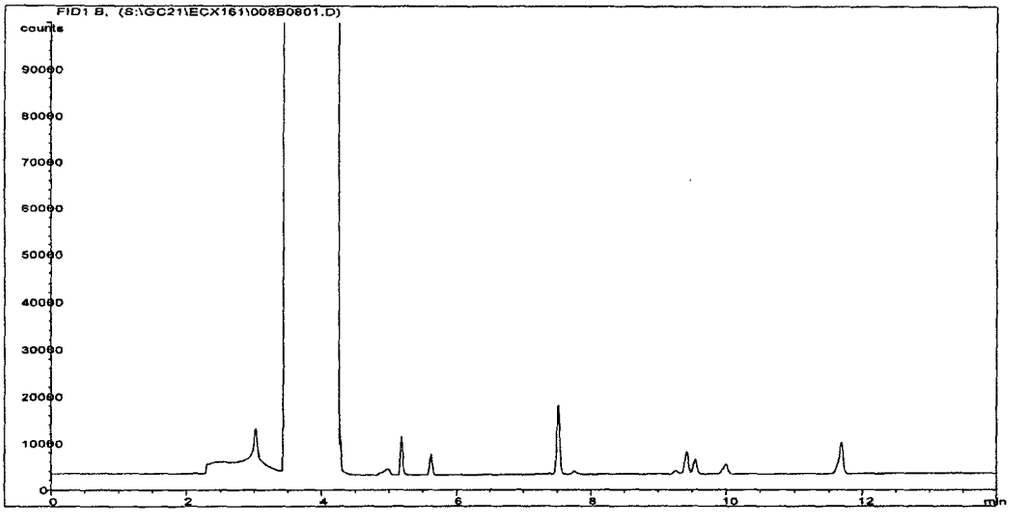
5. DISCUSSION

The detection system was found to have acceptable linearity. The analytical procedure had acceptable recoveries of test material in test medium. A method of analysis was validated and proven to be suitable for use.

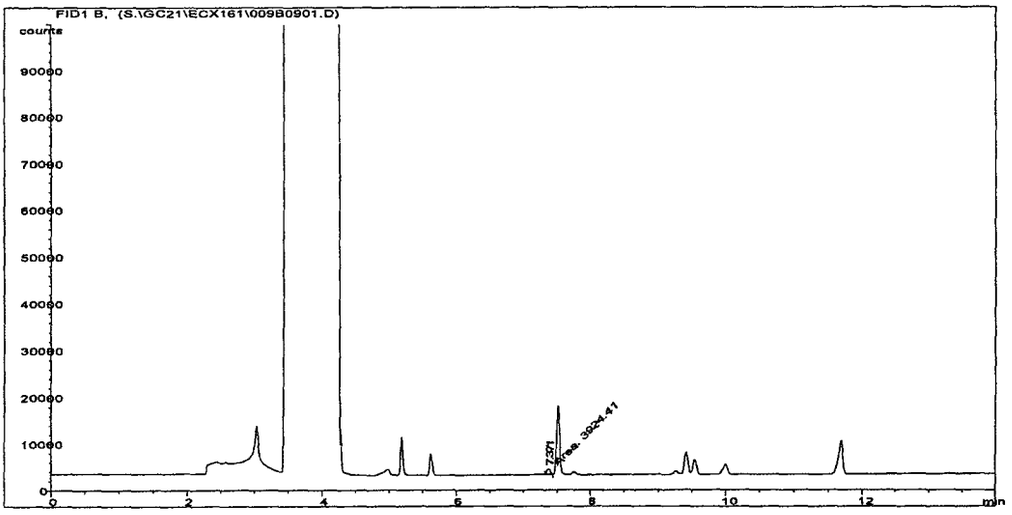
* Calculated on the basis of molar ratio: 116 mg tert-butyl acetate yields 74 mg tert-butyl alcohol on complete hydrolysis.
LOQ = Limit of quantitation

tert-Butyl acetate: ALGAL INHIBITION TEST

Appendix 2 (continued) Verification of Test Concentrations



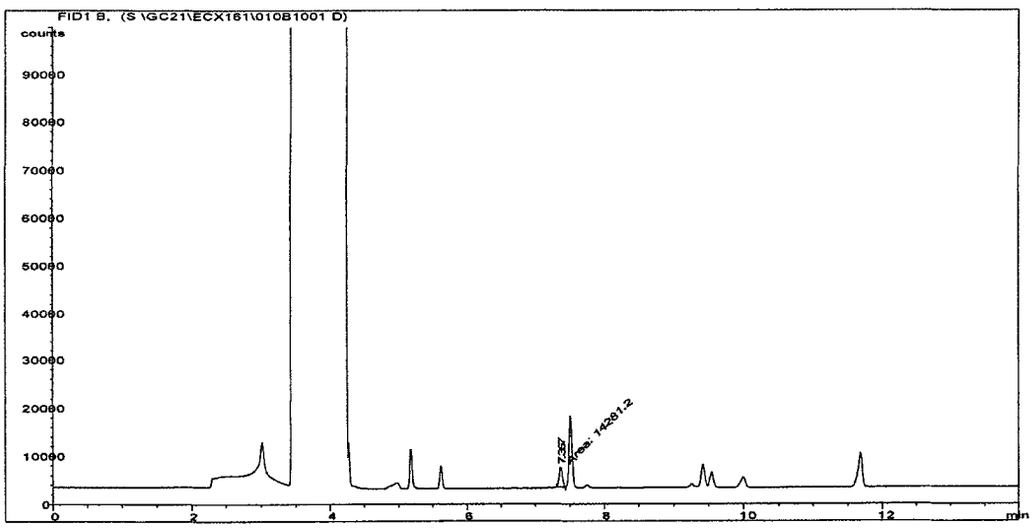
Control Sample 0 Hours



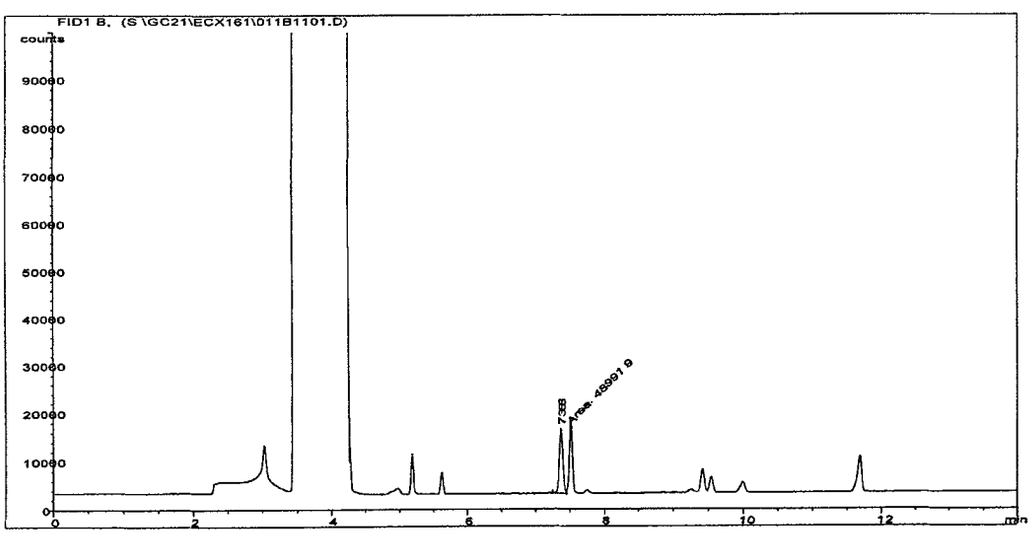
Test Sample 10 mg/l 0 Hours

tert-Butyl acetate: ALGAL INHIBITION TEST

Appendix 2 (continued) Verification of Test Concentrations



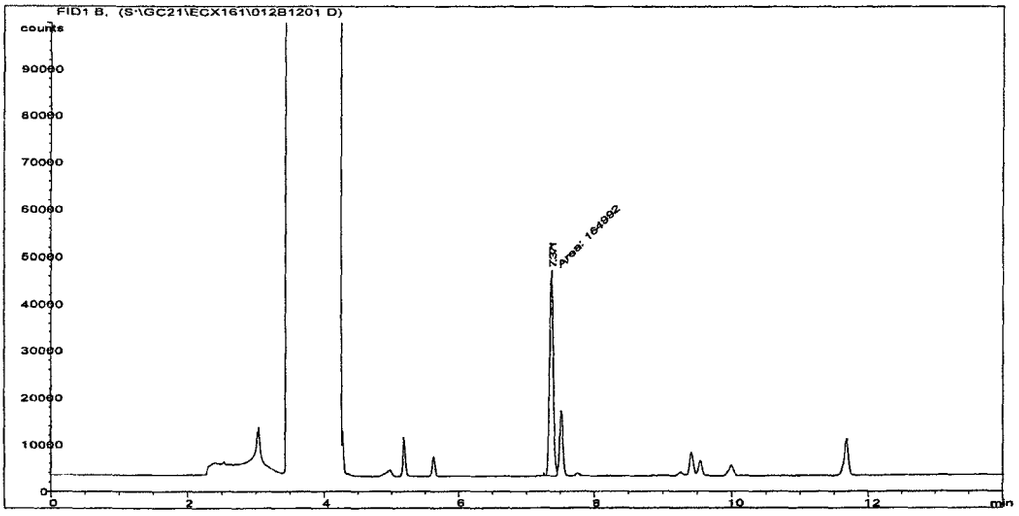
Test Sample 32 mg/l 0 Hours



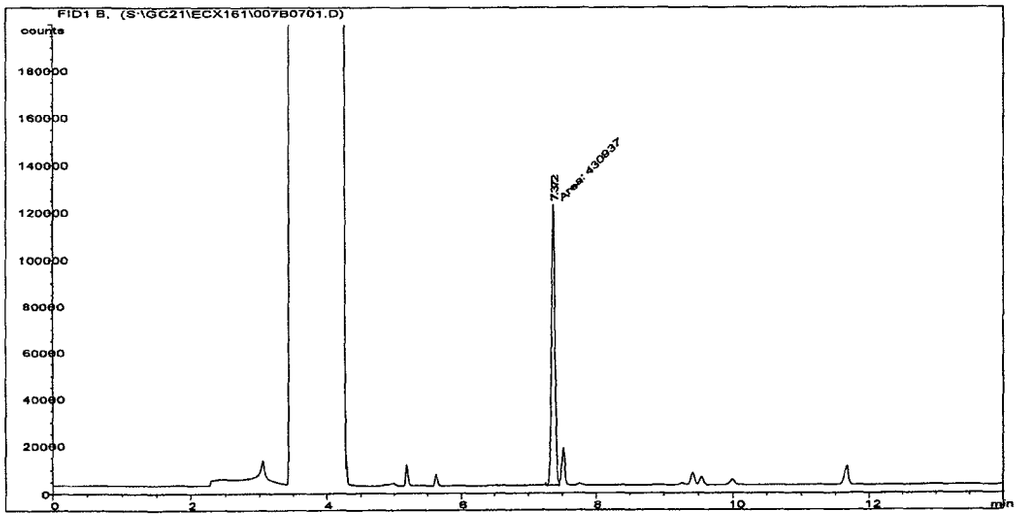
Test Sample 100 mg/l 0 Hours

tert-Butyl acetate: ALGAL INHIBITION TEST

Appendix 2 (continued) Verification of Test Concentrations



Test Sample 320 mg/l 0 Hours

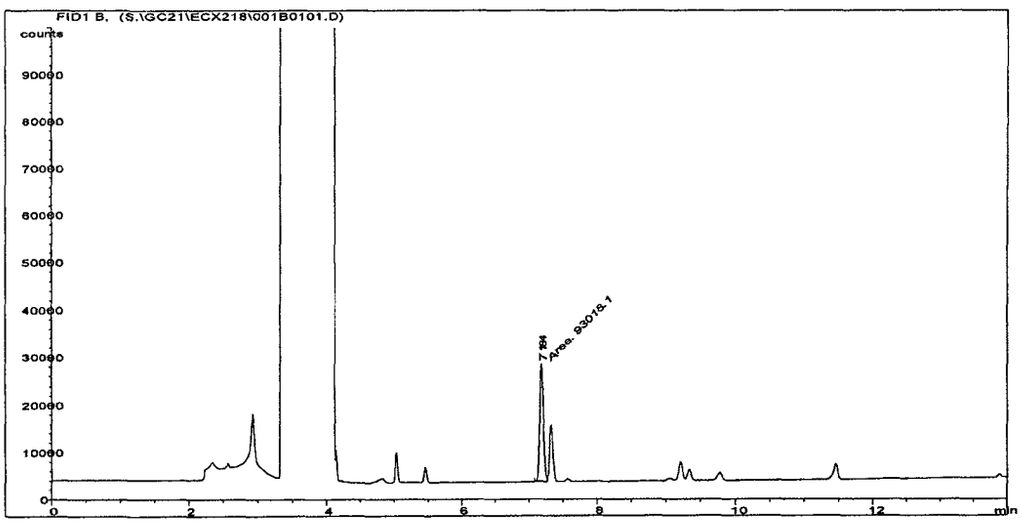


Test Sample 1000 mg/l 0 Hours*

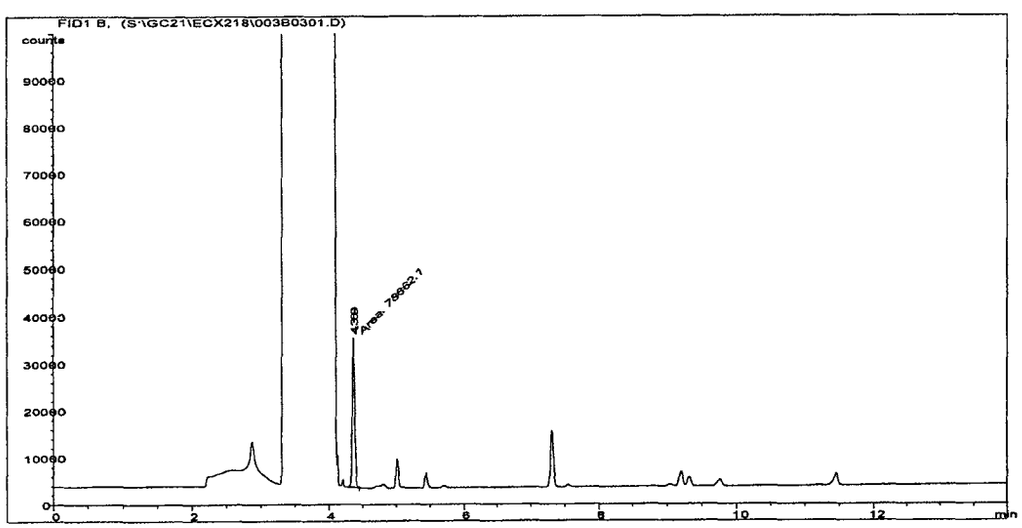
* Shown at half vertical scale of standard for clarity

tert-Butyl acetate: ALGAL INHIBITION TEST

Appendix 2 (continued) Verification of Test Concentrations



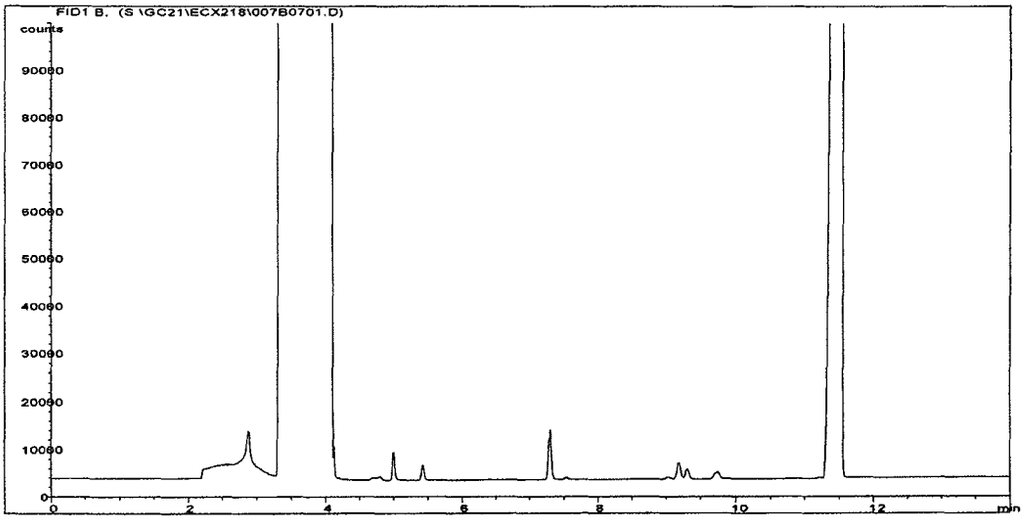
Test Material Standard 50 mg/l 96 Hours



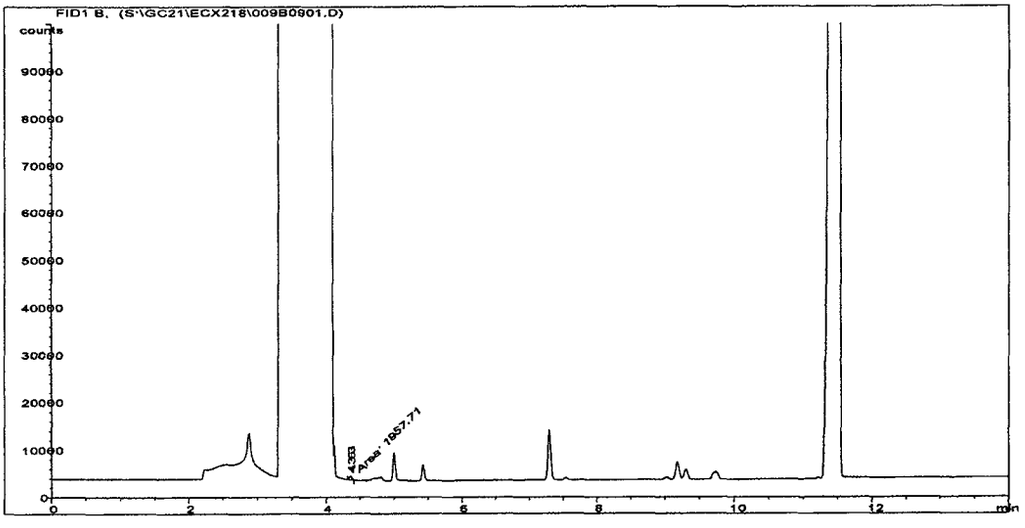
Degradant Standard 50 mg/l 96 Hours

tert-Butyl acetate: ALGAL INHIBITION TEST

Appendix 2 (continued) Verification of Test Concentrations



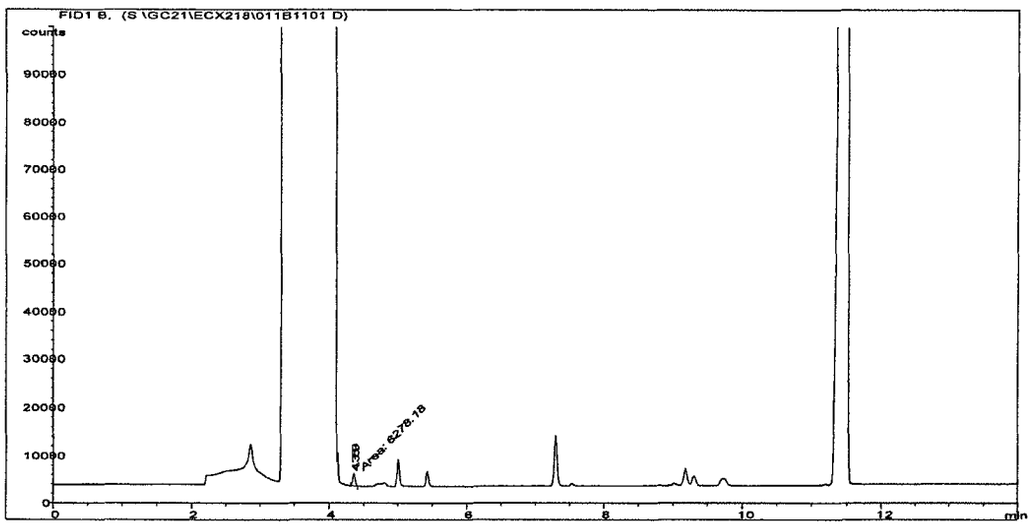
Control Sample 96 Hours



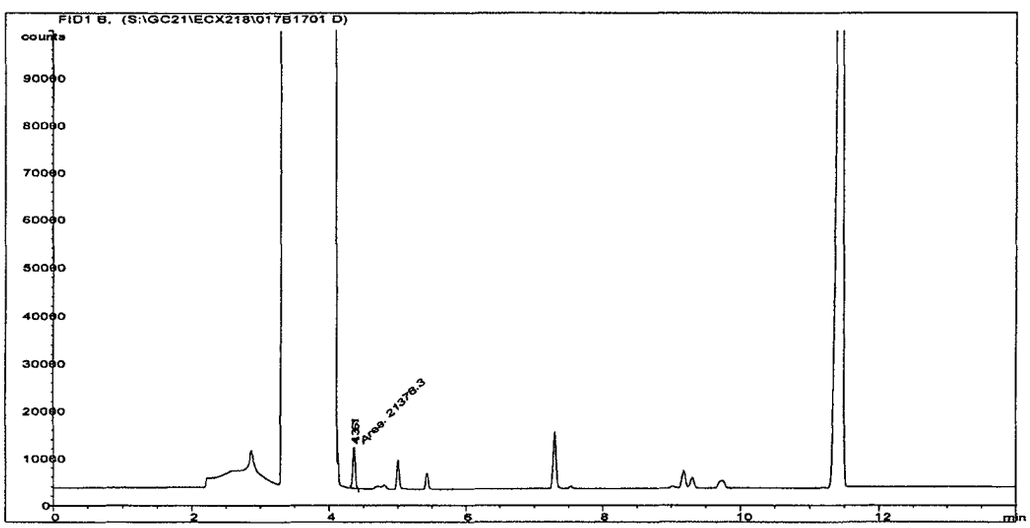
Test Sample 10 mg/l 96 Hours

tert-Butyl acetate: ALGAL INHIBITION TEST

Appendix 2 (continued) Verification of Test Concentrations



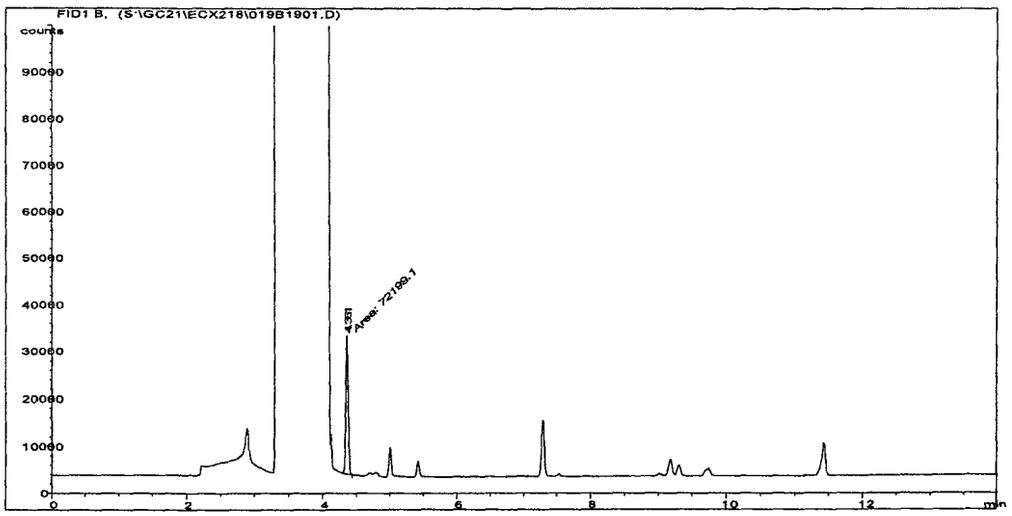
Test Sample 32 mg/l 96 Hours



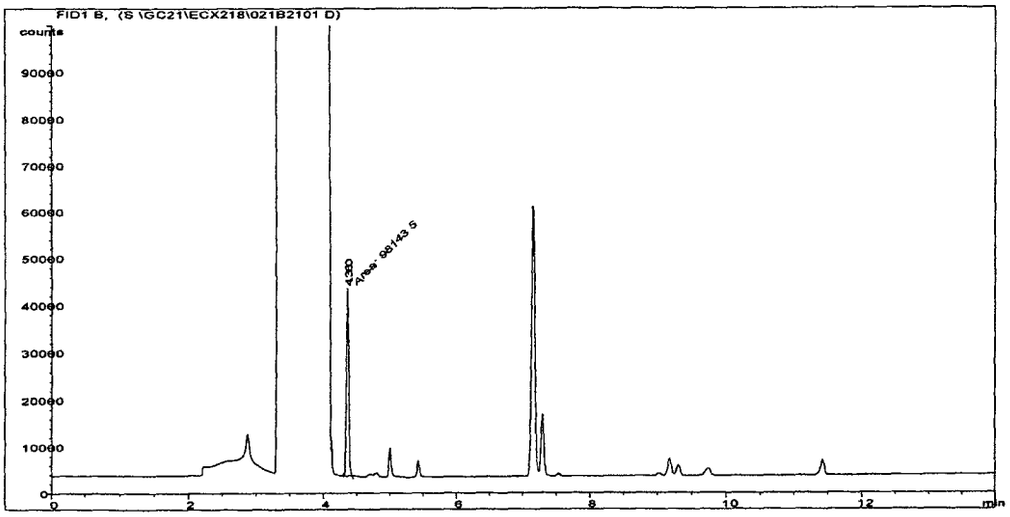
Test Sample 100 mg/l 96 Hours

tert-Butyl acetate: ALGAL INHIBITION TEST

Appendix 2 (continued) Verification of Test Concentrations



Test Sample 320 mg/l 96 Hours



Test Sample 1000 mg/l 96 Hours

Appendix 3 Statement of GLP Compliance in Accordance with Directive 2004/9/EC

DH Department of Health

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE UNITED KINGDOM

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE
IN ACCORDANCE WITH DIRECTIVE 2004/9/EC

LABORATORY	TEST TYPE
SafePharm Laboratories Ltd. **Shardlow Business Park** **London Road** **Shardlow** **Derby** **DE72 2GD**	**Analytical Chemistry** **Environmental Fate** **Environmental Toxicity** **Mutagenicity** **Phys/Chem Testing** **Toxicology**

DATE OF INSPECTION

30th August 2005

A general inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above laboratory as part of the UK GLP Compliance Programme.

At the time of inspection no deviations were found of sufficient magnitude to affect the validity of non-clinical studies performed at these facilities.

Bryan J Wright 21/11/05.

Mr. Bryan J. Wright
Head, UK GLP Monitoring Authority